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Health Benefits of Ajwain

Integrating Network Pharmacology

Highlights

Chrysanthemum against Dry Eye

Active Substances and Mechanisms

Discovering Thoughts, Inventing Future

VOLUME 24 ISSUE 1 VERSION 1.0

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Integrating Network Pharmacology and Molecular Docking to Identify the Active Substances and Mechanisms of Chrysanthemum against Dry Eye

By Zhang Yanxue, Tang Yu, Wu Kai & Yao Xiaolei
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Abstract- *Background:* Dry eye is the most common ocular surface disease and is widely treated with chrysanthemum, an herbal medicine known for its anti-inflammatory mechanism. However, the active ingredients, potential targets, and pathways of chrysanthemum in treating dry eye have not been clearly identified.

Objective: The objective of this study is to explore the bioactive components and potential mechanism of chrysanthemum on dry eye using network pharmacology and molecular docking.

Methods: We used the TCMSP database to search and screen the active ingredients of chrysanthemum and their related targets. Targets associated with dry eye were collected and screened using the Genecards database.

Keywords: *chrysanthemum, dry eye, chinese medicine, network pharmacology, molecular docking.*

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Results: We collected 20 potential active compounds and 220 component targets of chrysanthemum, as well as 1564 targets of dry eye, of which 118 intersection targets were obtained. PPI network analysis identified 5 key targets: TP53, JUN, MAPK3, MAPK1, and AKT1. These targets were mainly involved in biological processes such as apoptotic signaling pathway, response to oxygen levels, regulation of inflammatory response, regulation of cellular response to stress and aging. Enrichment analysis of KEGG pathways revealed IL-17 signaling pathway, PI3K-Akt signaling pathway, HIF-1 signaling pathway, NF-kappa B signaling pathway, Jak-STAT signaling pathway, Calcium signaling pathway, Leukocyte transendothelial migration pathway, Regulation of lipolysis in adipocytes pathway, Dopaminergic synapse pathway, etc.

Conclusion: Our study provides novel insights into the multiple targets and pathways involved in the effects of chrysanthemum in preventing dry eye. This data may aid in the development of new drugs and hypotheses for the treatment of dry eye.

Keywords: chrysanthemum, dry eye, chinese medicine, network pharmacology, molecular docking.

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I. Introduction

Dry eye is a common condition diagnosed in ophthalmic clinics that can significantly affect a patient's quality of life. The clinical manifestations of patients with dry eye include tear secretion disorder, along with itchiness, photophobia, blurred vision, and foreign body sensation in the eye [1]. The prevalence of dry eye is expected to continue to rise as the elderly population grows [2]. Currently, mainstream treatments for dry eye include tear substitutes, anti-inflammatory drugs, immunosuppressive drugs, and hormones. However, in cases of severe dry eye, tear supplementation may not be enough therapeutically. Additionally, some anti-inflammatory drugs and hormone replacement therapies may cause side effects [3]. Chinese medicine (CM), originating in China, regards the human body as an organic whole, and its therapies involve the interaction of multiple viscera and the adjustment of qi, blood, fluid, and humor [4]. CM is an independent and complete system, which has shown unique therapeutic effects with fewer side effects in the treatment of dry eye [5][6]. The use of CM has broad prospects in the field of dry eye treatment. In CM theory, dry eye is considered a white xerotic syndrome, first proposed in Compendium of Ophthalmology (Shen Shi Yao Han). White xerotic syndrome is believed to be a result of latent heat in the qi aspect, and dampness-heat in the spleen-lung collateral [7]. Chrysanthemum is clinically considered to possess the effects of dispersing wind-heat and pacifying the liver to improve vision. Therefore, chrysanthemum is a common drug for dry eye treatment in CM. The effectiveness of chrysanthemum against dry eye is not only applied in the clinical practice of CM but also in daily life, such as in medicated tea and medicated diets.

As molecular biology, pharmacology, and bioinformatics continue to develop, the modernization of CM is necessary [8][9]. Our group has explored the anti-inflammatory mechanism of total flavonoids of chrysanthemum on dry eye. By inhibiting the expression of IL-1β and TNF-α and promoting the synthesis of TGF-β1 mRNA and TGF-β1, the flavonoid-class active ingredients can reduce inflammation in a castrated male rabbit dry eye model[10]. However, the potential pharmacological mechanism of chrysanthemum and its

interaction with dry eye targets are still unclear and need further study.

Network pharmacology combined with molecular docking is a common method to study the effects of drugs on diseases. Network pharmacology is based on a network composed of chemicals, targets, and pathways, which combines computer science with medical science [11]. Its application in CM provides compelling evidence for the protein targets and potential mechanism of CM in the treatment of diseases. Additionally, molecular docking is a frequently used docking simulation method for predicting the optimal interaction of molecules. Our study aims to clarify the molecular targets and potential mechanism of chrysanthemum against dry eye by utilizing network pharmacology. Furthermore, we use molecular docking to simulate the binding mode of receptor proteins and ligands.

Chrysanthemum belongs to the Asteraceae family. In the Pharmacopeia of the National Health Commission of the People's Republic of China, chrysanthemum is listed as a dietary herbal medicine. Chrysanthemum contains a rich amount of flavonoids, which have superior free radical scavenging and antioxidant functions [12][13]. Additionally, it contains a number of other chemicals, such as phenylpropanoids, triterpenoids, and steroids [14]. What specific components of chrysanthemum act on dry eye and how do they work? We conducted this study to analyze the vital ingredients, important targets, and key pathways of chrysanthemum in dry eye treatment.

II. Materials and Methods

a) *Active components and targets retrieval of Chrysanthemum*

The main active components and corresponding targets of chrysanthemum were collected by using the Traditional Chinese Medicine Systems Pharmacology Database and Analysis Platform (TCMSP, http://tcmspw.com/tcmsp.php) with screening conditions "oral bioavailability (OB) ≥ 30%" and "drug-likeness (DL)≥ 0.18". TCMSP is a common ly used, comprehensive botanical platform that can retrieve related compounds, related protein targets, and their pharmacokinetic properties [15]. Next, the UniProt database was utilized to obtain the protein sequence of humans. The protein names and annotations were standardized and matched with chrysanthemum component targets using Excel. After that, the targets of unmatched genes were searched again with TCMSP. All matching genes were considered to be the targets of chrysanthemum.

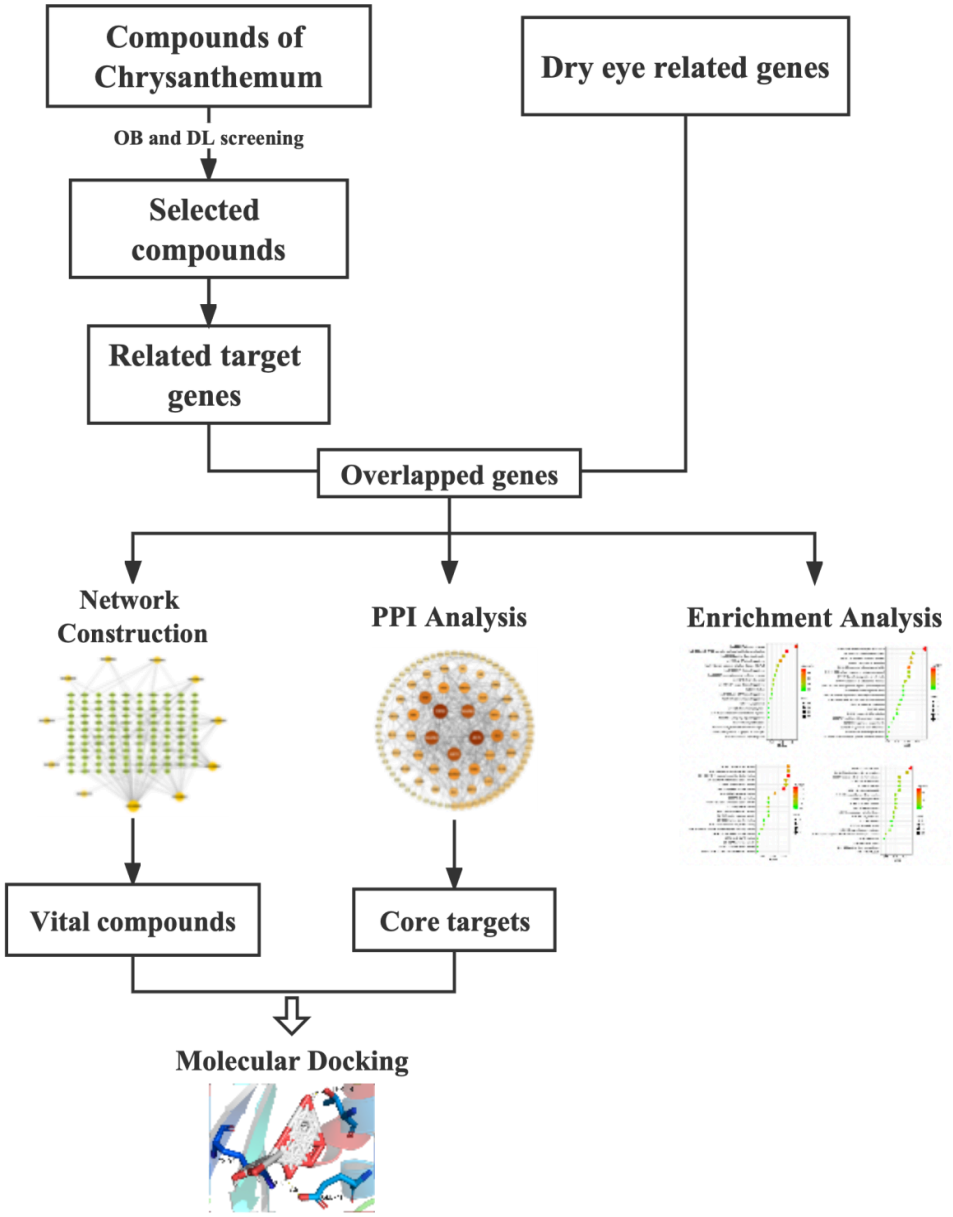

Fig.1: Flow chart of the experimental design

b) *Dry eye related targets retrieval and intersection targets visualization*

The related targets of dry eye were searched for in the GeneCards database using the keywords "dry eye" and "xerophthalmia" [16]. The relevance scores of the search result were ranked and the median was calculated to set the screen-out threshold score at 3 (≥3). The targets of dry eye identified, along with the chrysanthemum component targets, were inputted into VENNY 2.1.0 (https://bioinfogp.cnb.csic.es/tools/venny/index.html), and the intersection of drug targets and disease targets was collected as the potential targets of chrysanthemum for treating dry eye. The resulting chrysanthemum-dry eye target Venn diagram was drawn. The data for the chrysanthemum vital components and the intersection targets were then imported into Cytoscape 3.8.2 to construct the network of vital compound-disease interaction targets [17].

c) *PPI network constructing of chrysanthemum and dry eye*

118 intersection targets were imported into the STRING platform (https://cn.string-db.org) [18]. The protein organism was configured as "Homo sapiens" and the minimum required interaction score was set to the highest confidence (0.900). The protein-protein interaction (PPI) network was constructed with the disconnected nodes hidden. Import the tsv suffix file into Cytoscape 3.8.2 to draw a clearer PPI network. Additionally, a histogram should be plotted for the top 25 proteins based on their in-degree value.

d) GO and KEGG pathway enrichment analysis

The 118 intersection targets of chrysanthemum and dry eye were uploaded to Metascape database (https://metascape.org). Custom analysis was performed with Gene Ontology (GO) and KEGG pathway enrichment under the condition of $P \leq 0.01$. The enrichment results were imported into an online bioinformatics platform (http://www.bioinformatics.com.cn) to draw the relevant enrichment bubble plots.

e) Molecular docking

We chose the top 5 proteins ranked by degree as protein receptors. The 3D structure of TP53, JUN, MAPK3, MAPK1 and AKT1 were downloaded from PDB database (https://www. rcsb.org). Then, we use PyMOL 2.5.2. software to remove the organics and solvent. 3D structures of the top 4 vital compounds were downloaded from PubChem database (https://pubchem.ncbi.nlm.nih.gov). Additionally, molecular docking was performed using Autodock-Vina. TP53, JUN, MAPK3, MAPK1 and AKT1 were docked with quercetin (MOL000098), kaempferol (MOL000422), luteolin (MOL000006) and acacetin (MOL001689) respectively. The affinity score smaller than 0 indicates that the ligand and receptor can bind spontaneously. It is generally believed that the components with lower scores are the active ones interacting with the protein. Matrix heatmap was plotted by http://www.bioinformatics.com.cn, a free online platform for data analysis and visualization. At last, the docking results were analyzed by using PyMOL 2.5.2.

III. Result

a) Components and the corresponding targets of chrysanthemum

Twenty active components were obtained from chrysanthemum by using screening conditions of OB≥30% and DL≥0.18, including acacetin, linarin, chryseriol, isorhamnetin, kaempferol, and others. The corresponding targets were identified by searching for the chemical names and matching them with the protein sequence of human. After filtering out the remaining unmatched items, we obtained 220 targets of chrysanthemum components.

Table 1: The active compounds of chrysanthemum

MOLID	Molecule Name	OB	DL
MOL001689	acacetin	34.97%	0.24
MOL001790	Linarin	39.84%	0.71
MOL003044	Chryseriol	35.85%	0.27
MOL000354	isorhamnetin	49.60%	0.31
MOL000422	kaempferol	41.88%	0.24
MOL005100	5,7-dihydroxy-2-(3-hydroxy-4-methoxyphenyl)chroman-4-one	47.74%	0.27
MOL000006	luteolin	36.16%	0.25
MOL000098	quercetin	46.43%	0.28
MOL000358	beta-sitosterol	36.91%	0.75
MOL001506	Supraene	33.55%	0.42
MOL001733	EUPATORIN	30.23%	0.37
MOL001755	24-Ethylcholest-4-en-3-one	36.08%	0.76
MOL001771	poriferast-5-en-3beta-ol	36.91%	0.75
MOL002881	Diosmetin	31.14%	0.27
MOL004328	naringenin	59.29%	0.21
MOL005229	Artemetin	49.55%	0.48
MOL007326	Cynarin(e)	31.76%	0.68
MOL011319	TruflexOBP	43.74%	0.24
MOL011802	(24r)-saringosterol	39.36%	0.79
MOL011816	[(1S,5S,7S)-7-acetoxy-5-isopropenyl-2,8-dimethylene-cyclodecyl]acetate	37.02%	0.19

b) Candidate targets associated with Dry eye and chrysanthemum-dry eye intersection targets

After filtering out the targets with a relevance score smaller than the median (≥3), we obtained 1564 dry eye targets. Matching them with the chrysanthemum component targets resulted in a compound-target network of chrysanthemum on dry eye, which revealed 118 matched targets (as shown in Fig. 2) and 11 corresponding compounds. The interaction targets network (as shown in Fig. 3) highlighted the most vital four compounds: quercetin (MOL000098, 44 targets), kaempferol (MOL000422, 21 targets), luteolin (MOL000006, 19 targets), and acacetin (MOL001689, 11 targets).

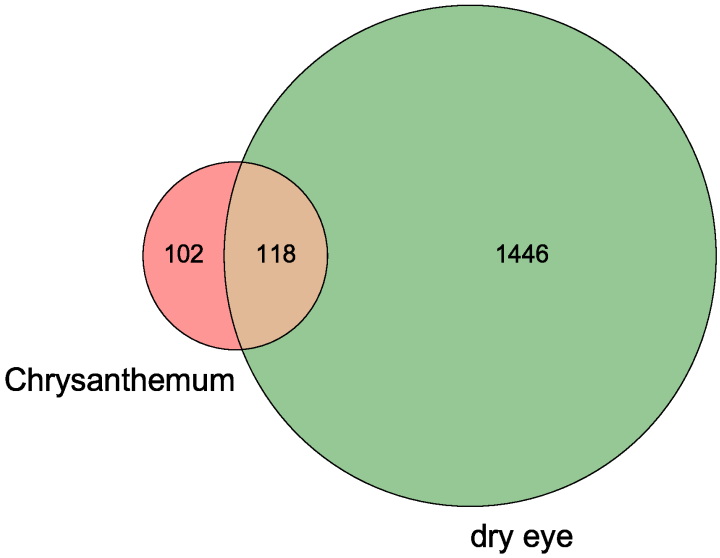

Fig. 2: Venn diagram of active compound targets of Chrysanthemum and related targets of dry eye.

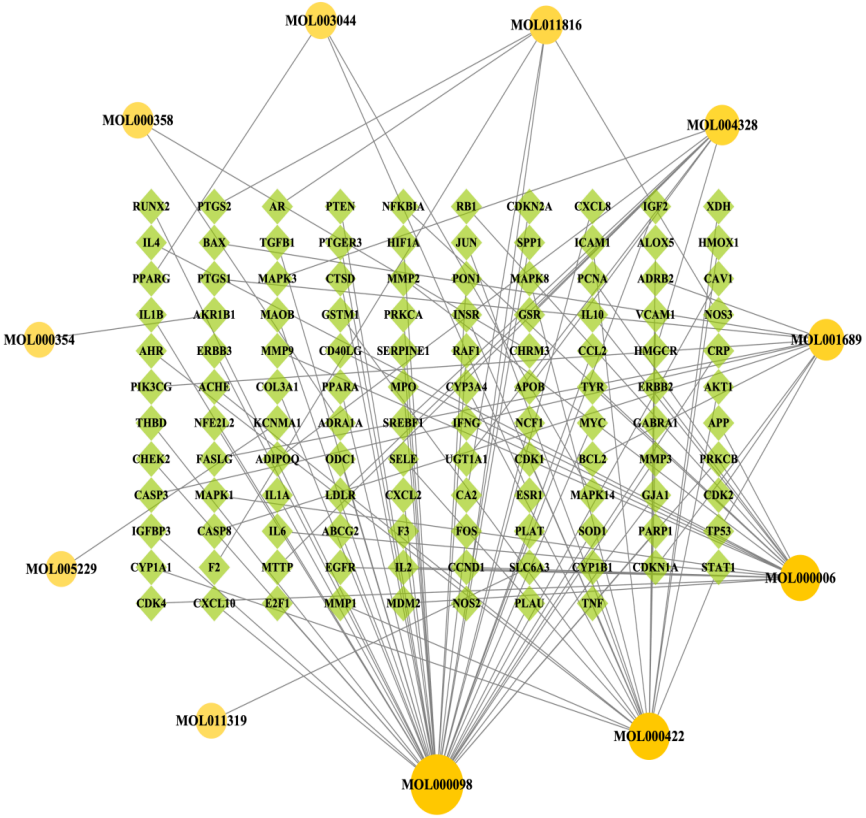

Fig. 3: The compound-target network of Chrysanthemum on dry eye. Yellow solid circles represent compounds and green diamonds represent targets.

c) PPI network analysis

The PPI network (Fig. 4) displays 118 nodes and 467 edges, with an average node degree of 7.92. The PPI enrichment p-value is <1.0e-16. The node degree indicates the number of edges of the target line. The higher the node degree, the more likely it is to be a key target of functional ingredients and play a significant role in the network. The nodes in the figure represent protein targets, and the edges represent protein-protein interactions. The more lines, the closer the relationship between targets. To illustrate this, the top 25 genes with the highest degree value were plotted in a histogram (Fig. 5), showing that TP53, JUN, MAPK3, MAPK1, and AKT1 are the most prominent proteins.

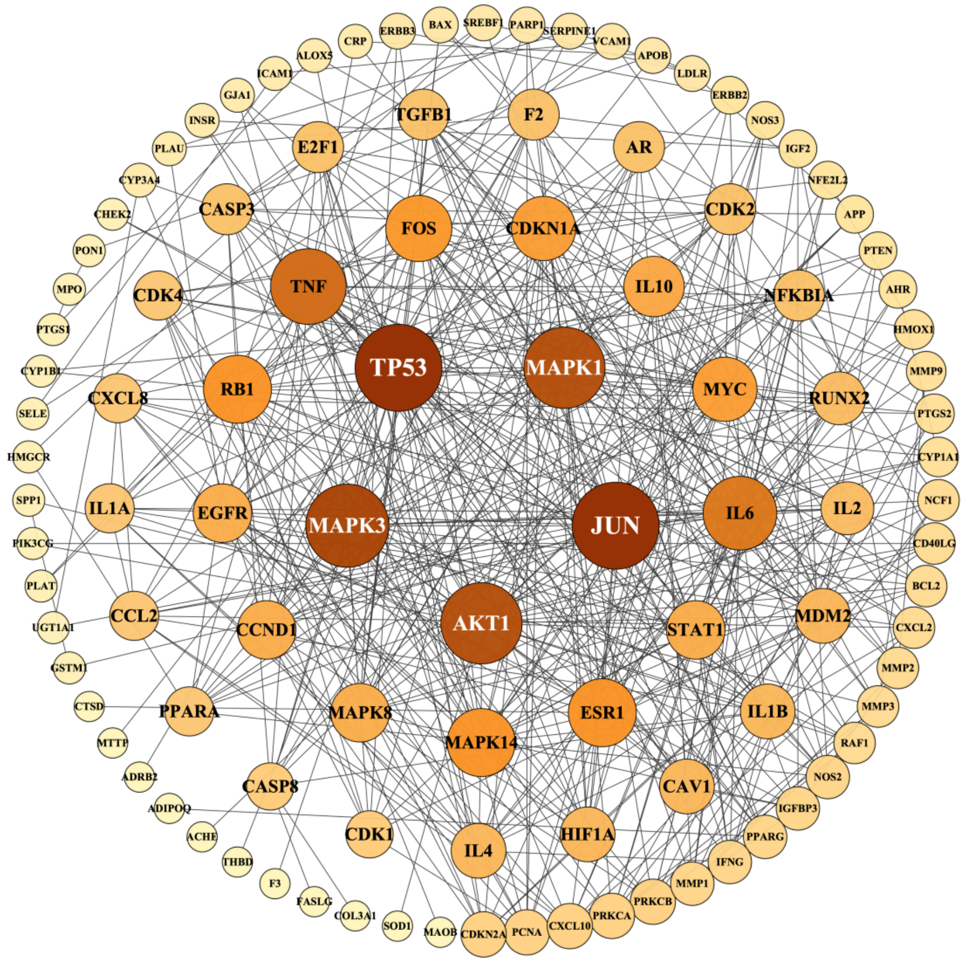

Fig. 4: Network diagram of protein-protein interaction (PPI)

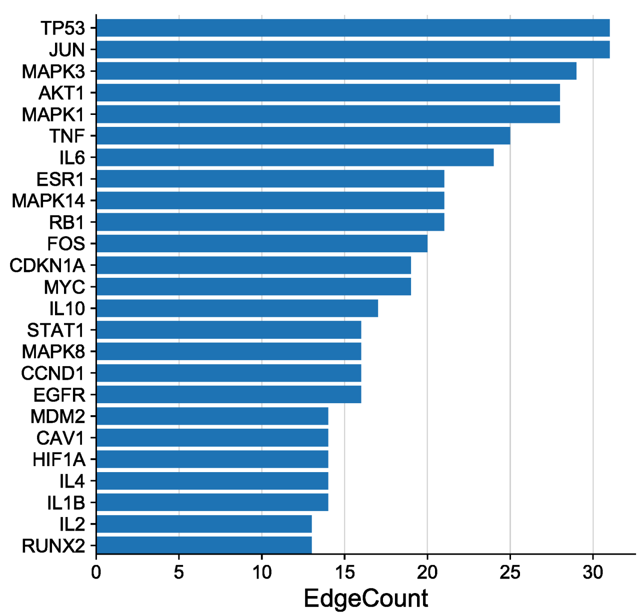

Fig. 5: Top25 targets by degree value

d) GO&KEGG pathway enrichment analysis

Enrichment result shows 431 cellular components (CC), 719 molecular functions (MF), 5365 biological processes (BP) and 455KEGG pathways. The top 20 significantly enriched entries in the results are arranged in an ascending order of p value (Fig. 6). The results showed important MF such as kinase binding, protease binding, cytokine receptor binding, DNA-binding transcription factor binding, kinase activity and cytokine activity; significant BP such as apoptotic signaling pathway, response to oxygen levels, regulation of inflammatory response, regulation of cellular response to stress and aging; important CC contains membrane, cytoplasm, endoplasmic reticulum, etc. The enrichment analysis results of KEGG pathways mainly manifested IL-17 signaling pathway (hsa04657), PI3K-Akt signaling pathway (hsa04151), HIF-1 signaling pathway (hsa04066), NF-kappa B signaling pathway (hsa04064), Jak-STAT signaling pathway (hsa04630), Calcium signaling pathway (hsa04020), Leukocyte transendothelial migration pathway (hsa04670), Regulation of lipolysis in adipocytes pathway (hsa04923) and Dopaminergic synapse pathway (hsa04728).

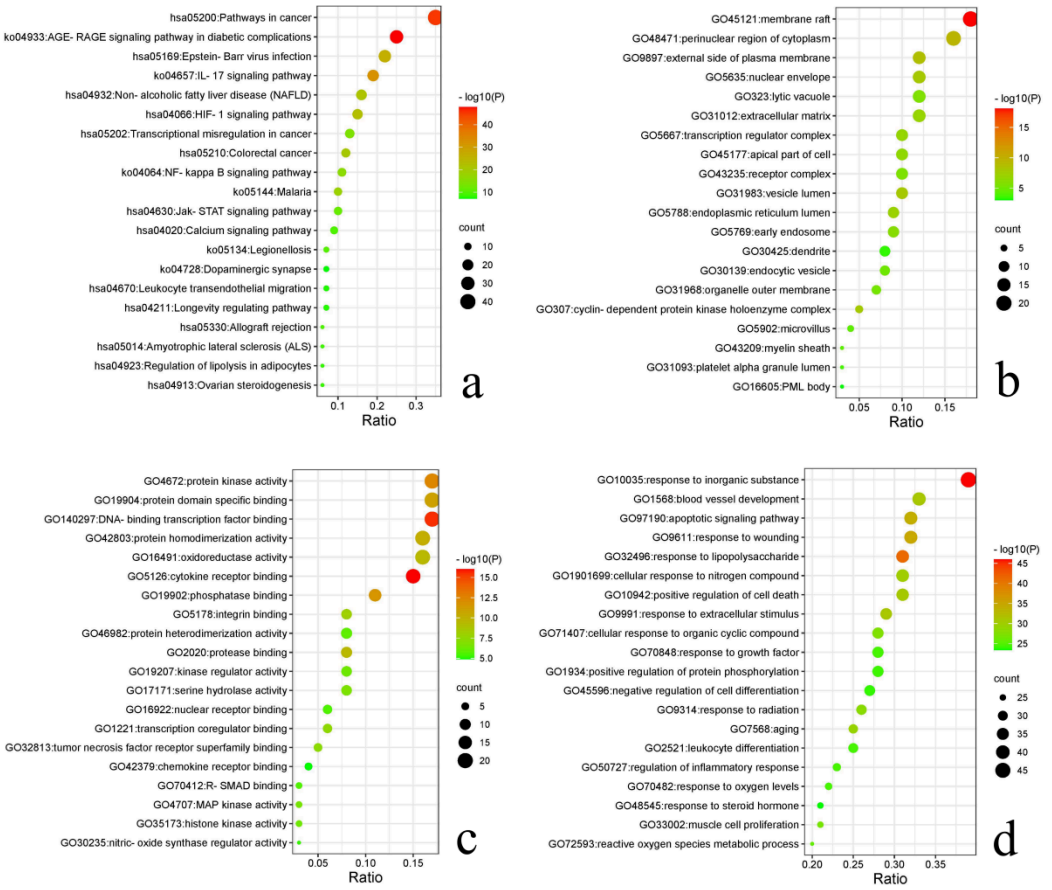

Fig. 6: Enrichment bubble plots of GO&KEGG pathway enrichment analysis
.a Enrichment analysis results of KEGG pathways; b Enrichment analysis results of CC; c Enrichment analysis results of MF; d Enrichment analysis results of BP. Size and color of the dots represent the degree of GO enrichment analysis.

e) Molecular docking results and analysis

The top 5 core targets were respectively docked onto the top 4 vital chemicals. All docking simulations show good combination states. The affiniy scores of TP53, JUN, MAPK3 and MAPK1 are slightly more ideal than AKT1. The representative ones in the docking results are TP53 with luteolin (-9kcal/mol), JUN with quercetin (-9.4kcal/mol), MAPK3 with quercetin (-10kcal/mol) and MAPK1 with quercetin (-9.5kcal/mol). These four receptors and their best docked ligands were picked out to draw the molecular docking diagram subsequently. Notably, MAPK3 also shows remarkable docking state with other 3 ligands. Its docking score with kaempferol, luteolin and acacetin are -9kcal/mol, -9.4kcal/mol, -9.2kcal/mol, respectively. The affinity scores heatmap and docking simulation images are shown in figure 7 and figure 8.

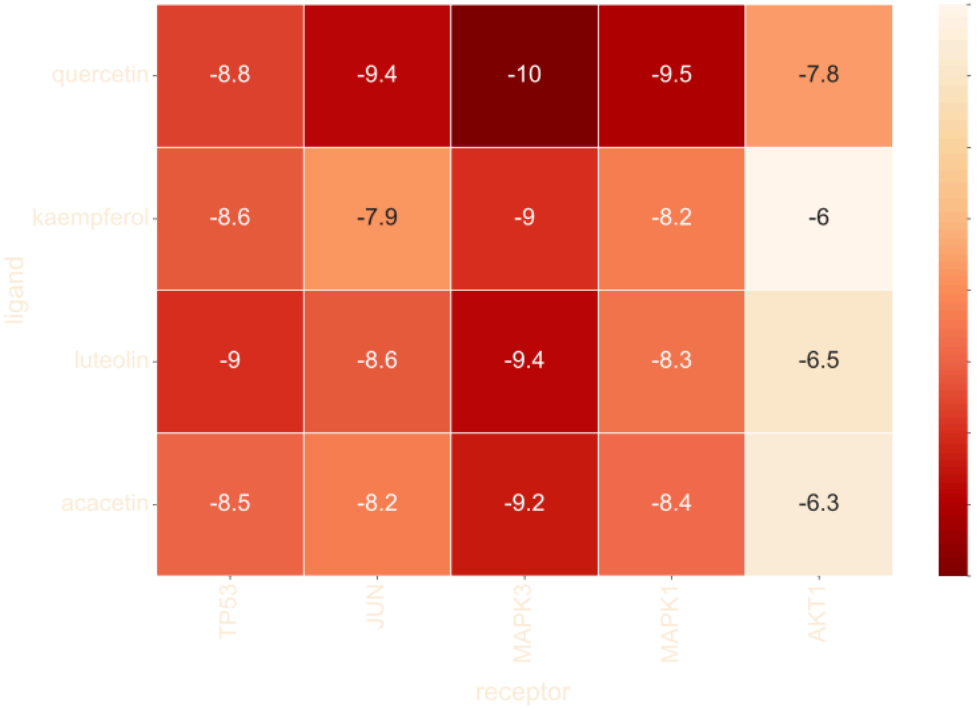

Fig. 7: The heat map of the affinity score

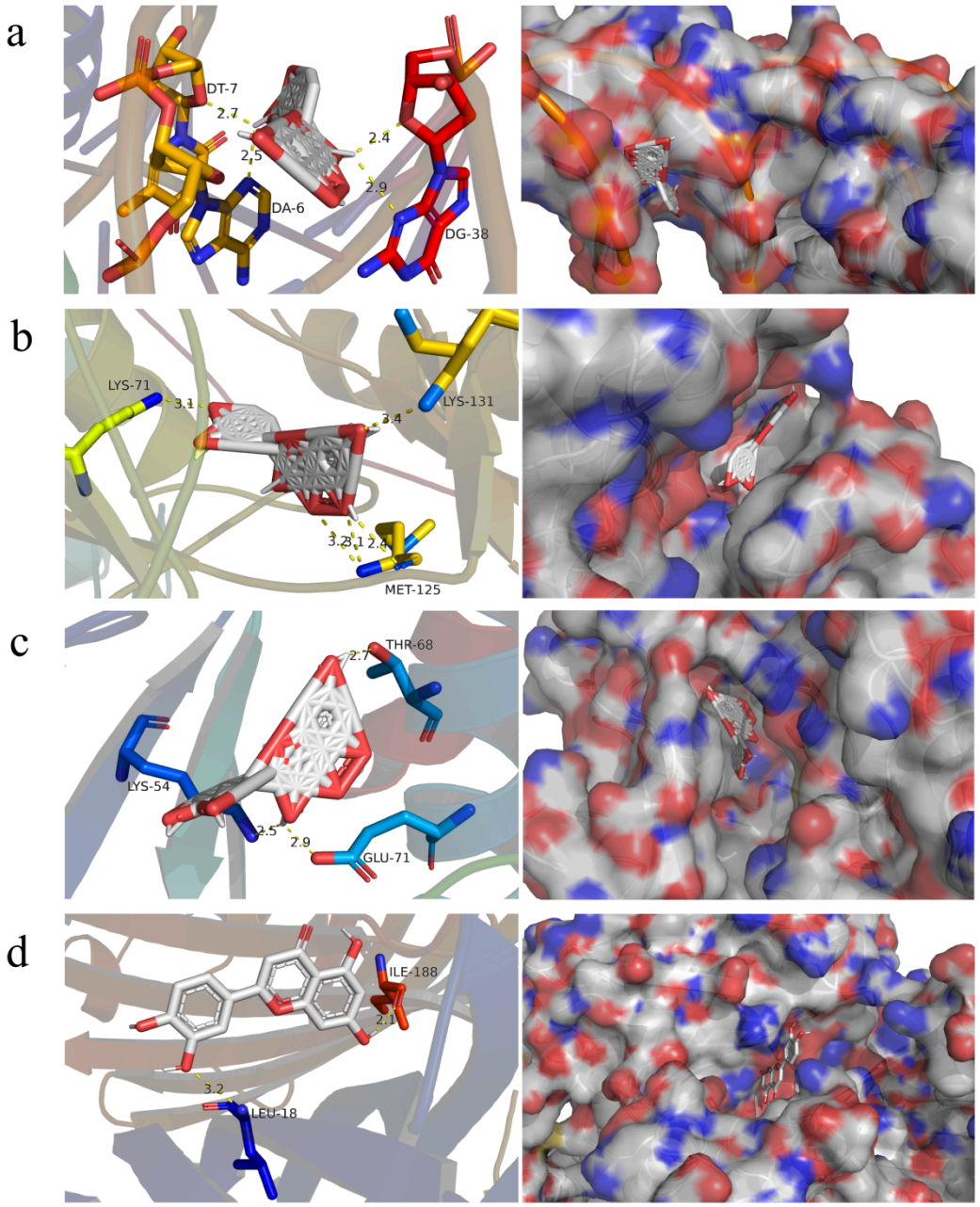

Fig. 8: The notable results of molecular docking analysis. aAction mode of quercetin with target JUN(PDB ID:5T01); bAction mode of quercetin with target MAPK3(PDB ID:4QTB); cAction mode of quercetin and MAPK1(PDB ID:7NR8); dAction mode of luteolin and TP53(PDB ID:7BWN)

IV. Discussion

In CM theory, chrysanthemum is believed to be effective in treating dry eye by dispersing wind-heat and pacifying the liver, which in turn improves vision. However, due to the complex composition of herbal medicine, it is often difficult to understand its underlying mechanisms. To address this issue, we used network pharmacology to establish a drug-target-disease network, which helped us to explore the active ingredients and therapeutic targets of chrysanthemum. Our results suggest that chrysanthemum can treat dry eye through the action of multiple targets and components, which is consistent with the manifold therapeutic effects of CM. Although chrysanthemum and chrysanthemum-containing formulas have been the subject of some clinical experiments[10][19], there is still much to learn about the combination of its components and the enrichment of its targets. Therefore, we believe that exploring the vital components and targets before experimental validation may be more conducive to research in this field.

It is widely recognized that inflammation is a central mechanism of dry eye, as supported by

numerous in vitro, in vivo, and human studies [20]. Inflammatory cytokines are the main culprits of dry eye-associated inflammation [21]. Moreover, research suggests that an abnormal immune response in the ocular system can also contribute to ocular surface injury, further exacerbating dry eye symptoms [22]. Additionally, oxidative stress is also a critical factor in the pathogenesis of dry eye. Oxidative stress mouse models have shown decreased tear secretion, leukocyte infiltration, and fibrosis [23]. Chrysanthemum's bioactive components have the effect of anti-inflammation, antibacterial, antifungal, anti-spirochete, anti-human immunodeficiency virus, and antioxidant [13].This provides theoretical support for the anti-inflammatory effect of chrysanthemum treating dry eye.

According to the degree ranking of targets interaction, the corresponding most important components are quercetin, kaempferol, luteolin and acetin, which all belong to the ranks of natural flavonoids. Previous studies have already shown that flavonoid compound has strong anti-inflammatory and antioxidant effects [19][24], which is consistent with our earlier research[10]. Quercetin's topical application can not only reduce the irregularity of the ocular surface, but also increases the amount of tears and goblet cell density [25]. However, quercetin's oral bioavailability is still controversial, so we need more information about its pharmacokinetics in experiments [26]. Intragastric kaempferol feeding can inhibit nlrp1 / NLRP3 inflammasomes and caspase8 through NF-κB and JNK pathway, so as to reduce the inflammatory damage of retinal ganglion cells in acute glaucoma mice model [27]. Kaempferol-contianing eye drops in rabbit dry eye model have also been proved to reduce corneal epithelial injury and increase tear secretion [28]. In the rat uveitis model, luteolin inhibits the inflammatory markers and activates NF-κB pathway, thereby reducing iris-ciliary body inflammation. The anti-inflammatory effect of 10 mg / kg luteolin being injected intraperitoneally was proved to be as strong as 1 mg / kg prednisolone [29]. Interestingly, Acacetin has shown therapeutic potential in inflammation, infections and other metabolic disorders [30][31][32]. However, there are few studies on its application in the field of Ophthalmology, which deserves our attention.

According to the our PPI network data as well as molecular docking results, we conclude that the core targets chrysanthemum regulating dry eye are TP53, JUN, MAPK3 and MAPK1, which all showed stable structure and high binding activity with the vital ingredients in molecular docking. TP53 cooperating with mitochondrial PPIF is involved in activating oxidative stress-induced necrosis, which damages the ocular surface and plays an important role in the mechanism of dry eye disease [33]. MAPK family is involved in many processes, such as cell proliferation, stress, inflammation, apoptosis and so on. Studies have proved the efficacy of p38 MAPK inhibitor on lacrimal gland secretion and neurotransmitter secretion in dry eye [34]. JUN protein is also related to cell aging induced by oxidative stress, however, its related studies in dry eye remain limited. Considering its good affinity scores with the ligands, it may serve as another latent therapeutic target of dry eye. Docking is broadly applied in the field of drug discovery and design as well as the identification of ligands binding to a target receptor [35]. We expect to see "pocket-like" docking simulation status. As can be seen in the docking image, the docking model reveals a good morphologic complementary between the receptors and ligands contact areas. Quercetin formed 4 hydrogen bonds with residue DT-6/7 and residue DG-38 of the JUN protein that shows a good binding affinity (-9.4kcal/mol).Whilst it could form 5 hydrogen bonds with residue LYS-71/131 and residue MET-125 of MPK3 which both show good binding affinity(-10kcal/mol). It also could form 3 hydrogen bonds with residue LYS-54, residue THR-68 and residue GLU-71 of MPK1 showing a good binding affinity (-9.5kcal/mol). Meanwhile, Luteolin could form 2 hydrogen bonds with residue ILE-188 and residue LEU-18 of TP53, displaying a good binding affinity (-9kcal/mol). These ligand-receptor combinations need to be further studied theoretically and experimentally.

The enrichment results have revealed several important KEGG pathways. The interleukin 17 (IL-17) family has been found to play a crucial role in both acute and chronic inflammatory responses. Topical application of IL-17 therapy has been shown to reduce ocular surface symptoms of tear evaporation and meibomian gland dysfunction [36]. The activation of the PI3K–AKT signaling pathway has also been found to have protective effects on injured RGCs [37]. Additionally, the NF-κB signaling pathway and JAK-STAT signaling pathway have been found to be critically involved in cellular stress and inflammation, and have been the focus of numerous studies in ophthalmology and Sjögren's syndrome[38][39][40]. It is worth noting that inflammation is also the primary mechanism of Sjögren's syndrome, which can trigger dry eye. Studies have shown that Dll4/Notch Signaling and HIF-1α Stimulating lymphangiogenesis may protect lacrimal glands from dry eye-induced inflammation by helping to clear immune cells in the lacrimal glands[41].The deficiency of HIF-1α enhances the recruitment of inflammatory cells to lacrimal glands[42]. Our enrichment analysis has also identified some core functions and processes, including oxidoreductase activity and response to oxygen levels, which have drawn our attention. These results provide guidance for the direction of our subsequent experimental research and may provide new insights for the treatment of dry eye.

V. Conclusion

The results of our enrichment analysis and molecular docking revealed that quercetin and luteolin are the important components of chrysanthemum in the treatment of dry eye, followed by kaempferol and acacetin. These components have shown promising anti-inflammatory and antioxidant properties. Interestingly, the anti-inflammatory mechanism of acacetin in ophthalmology has not been studied extensively, and further research is needed to explore its potential therapeutic effects. Our molecular docking studies showed that TP53, JUN, and MAPK3/1 are the core targets of chrysanthemum in the treatment of dry eye. Quercetin with JUN, quercetin with MAPK3/1, and luteolin with TP53 were identified as the most stable docking combinations, indicating their potential as effective therapeutic combinations for the treatment of dry eye. These findings provide valuable insights for further experimental studies on the potential therapeutic effects of chrysanthemum in the treatment of dry eye.

Declarations

Ethics approval and consent to participate

Not applicable

Availability of data and materials

All data generated or analysed during this study are included in this published article. All data generated or used during the study are available from the corresponding author by request.

Competing interests

The authors declare no conflict of interests.

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Authors' contributions

Yanxue Zhang wrote and revised the manuscript. Xiaolei Yao designed and adjusted structure of the manuscript. Yu Tang, Kai Wu collected and analyzed the data. All authors discussed the results and revised the manuscript.

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Abbreviations

CM: Chinese Medicine; TCMSP: Traditional Chinese Medicine Systems Pharmacology; OB: Oral Bioavailability; DL: Druglikeness; GO: Gene ontology, KEGG: Kyoto Encyclopedia of Genes and Genomes; PPI: Protein-protein interaction; BP: Biological Processes; CC: Cell Components; MF: Molecular Functions; TP53: Cellular tumor antigen p53; JUN: Transcription factor AP-1; MAPK3:Mitogen-activated protein kinase 3; MAPK1:Mitogen-activated protein kinase 1; IL-17: Interleukin-17; HIF-1 :Hypoxia-inducible factor 1; AKT1: RAC-alpha serine/threonine-protein kinase; PI3K-Akt:The phosphatidylinositol 3'-kinase-serine/threonine-protein kinase; Jak-STAT: Janus kinase/signal transducers and activators of transcription; NF-kappa B:Nuclear factor-kappa B

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Health Benefits of Ajwain (Tracyspermum Ammi L.)

By Dr. Yasmeen Ansari

Abstract- Ajwain is highly valued in Unani as a gastrointestinal remedy and as an antiseptic. It is mixed with salt and hot water and brought after meals to alleviate bowel ache or colic and to enhance digestion. Ajwain is also a conventional treatment for cholera and fainting spells. Westerners typically use it for coughs and throat problems. It is also a component in mouthwashes and toothpastes because of its antiseptic homes. The volatile oil present within the seeds of ajwain is one of the predominant components accountable for imparting a normal taste, attributable to the presence of thymol. It also incorporates a cumene and terpene. The methanolic extracts of ajwain seeds possess herbal antioxidant properties. However, the acetone extract showed higher antioxidative hobby for linseed oil compared to synthetic antioxidants consisting of butylated hydroxy toluene and butylated hydroxy anisole. Ajwain oil exhibited a vast spectrum of fungitoxic behavior towards all tested fungi. Immediate research should focus on validating the antioxidant capability of herbs and spices after harvesting, in addition to testing their consequences on markers of oxidation.

Keywords: *ajwain, unani, traditional.*

GJMR-B Classification: *NLMC Code: QV766*

Strictly as per the compliance and regulations of:

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I. Introduction

Known as Ajwain, Trachyspermum ammi (L.) Sprague is an annual herbaceous plant belonging to the distinctly valued medicinally vital own family, Apiaceae [1]. It is stated that the herb is extensively grown in arid and semi-arid regions in which the soil involve excessive quantity of salts [2]. Ajwain has an erect and striate stem regarding glabrous or minutely pubescent properties which may also grow as much as 90 cm tall [3]. Ajwain is widely disbursed and cultivated in various regions such as Iran, Pakistan, Afghanistan, and India in addition to Europe at the same time as it is indigenous to Egypt [4]. The herb is commonly grown in October–November and must be harvested in May–June [5, 6]. Usually grayish brown seeds or fruits of Ajwain are taken into consideration for clinical and nutritional purposes [5].

Oral application of seed changed into pronounced to be beneficial for paralysis, tremor and palsy as well as different neural issues within the discipline of neurology [6]. Persian practitioners additionally applied the eye and ear drop formulated from seeds of Ajwain with a purpose to control the infected conditions and accurate the auditory weak point [7]. In the field of respiration, Ajwain become stated to be powerful on cough, pleurisy and dysphonia [8]. Fruits were widely administered for liver spleen in addition to gastrointestinal problems such as nausea, vomiting, reflux, belly cramps and lack of appetite [6]. They have been additionally stated to be useful in belly problems and own stimulant and carminative properties [7]. Ajwain changed into stated as an anthelmintic medicinal drug and also antidote for diverse natural poisonous agents [7]. It became also believed to be beneficial for dissolving the calculi and stones if focused on wine. Persian practitioners also taken into consideration the seeds as an aphrodisiac, galactagogue and diuretic agent [6].

II. Discussion

Ajwain (Trachyspermum ammi) is a plant that produces small, seed-like fruits much like caraway and cumin. It comes from the Apiaceae circle of relatives, which is a collection of plant life that consists of celery, caraway, coriander, fennel, parsley, and parsnips. It goes by way of many different names, including carom seed, bishop's weed, and ajowan caraway.

Ajwain extensively utilized every now and then as an element in barbered, a spice combination desired in Eritrea and Ethiopia [9, 10]. Ajwain is an annual herbaceous plant having 30-70 cm (1-2 ft) height, with feathery leaves and crimson plant life. Ripe seeds are dried and threshed [11,12] manually and/ or routinely. Ajwain seed (fruit) is said to have antifungal/ antibacterial, antiseptic and antithelminitic effects [13]. The essential phenolic compound thymol determined in ajwain has been pronounced to be an antispasmodic, germicide and antifungal agent [14]. The principle energetic elements of ajwain oil are phenols, particularly thymol (35-60%) and some carvacrol [15]. Both the phenols thymol and carvacrol are having antiseptic, expectorant and antitussive residences [16]. Thymol also has antiseptic pastime and carvacrol possesses antifungal homes.

Ajwain is not unusual in Indian food. It has a strong, sour flavor with an aroma just like thyme. The "seeds," which can be truely culmination, are usually dry-roasted or floor and used in spice mixtures. They also are utilized in Unani and Ayurvedic remedy to help treat severa problems. These are recuperation structures that involve the notion that your ordinary fitness and wellbeing rely upon a balance among your body, thoughts, and spirit.

a) Health Benefits [9-10]

Ajwain seeds have a small amount of oil in them known as ajwain oil. The oil includes thymol, a phenol that offers the fruit its thyme-like scent. Thymol is usually

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used to treat digestive problems. It also has antifungal and antibacterial residences.

Here are some of the fitness benefits that ajwain has to offer:

Digestive Health

Active enzymes in ajwain enhance the drift of belly acids, that can help to relieve flatulence indigestion and gasoline. The plant also can assist to treatgastric ulcers as well as sores in the esophagus, belly, and intestines.

Figure 1: Health Benefits of Ajwain

Infection Prevention

Many of the important oils in ajwain, most substantially thymol and carvacrol, can assist to combat the increase of micro organism and fungi. They may help to combat micro organism like salmonella and E. Coli, which could lead to food poisoning and other belly problems.

Lower Blood stress

Research in rats indicates that thymol in ajwain may act to preserve calcium from entering the blood vessels on your heart, supporting to decrease blood pressure.

Cough and Congestion Relief

Ajwain can offer comfort from coughing as well as clean mucus out of your nostril, both of which make respiration less difficult. It may additionally assist to widen the bronchial tubes, that can help people with asthma.

Toothache Relief

Due to the anti inflammatory residences of thymol and different important oils, ajwain can help to lessen ache associated with toothaches. Thymol may also assist to enhance your oral fitness via preventing micro organism and fungi in the mouth.

Arthritis Pain Relief

Ajwain also can assist to assuage pain and swelling. Crushed fruit may be made into a paste and applied to the skin at the joints to treat arthritis ache. Alternatively, you can fill your tub with warm water and upload a handful of seeds for a soothing tub.

Medicinal and Pharmacological Properties

Antimicrobial activity: Bacteria like gram-fine and gram-poor may be suppressed by means of nigella essential oil. It exhibited strong antimicrobial activity in opposition to Salmonella typhi, Pseudomonas aeruginosa and others. Comparatively higher sensitivity against gram-

tremendous micro organism Staphylococcus aureus and Vibrio cholera become located to be stronger than gram bad micro organism. Staphylococcusaureus, Staphylococcuspyogenes and Staphylococcusviridans are greater liable to Nigella sativa.[17]

Antifungal hobby: Methanolic extracts of Nigella have the most powerful antifungal effect accompanied by the chloroform extracts towards one of a kind traces of Candida albicans. An intravenous inoculum of Candida albicans produced colonies of the organism in the liver, spleen and kidneys. Treatment of mice with the plant extract 24h after the inoculation prompted a vast inhibitory impact on the growth of the organism in all organs studied. Khan et al. In 2003 pronounced that the aqueous extract of Nigella seeds well-known shows inhibitory impact against candidiasis in mice. A five-fold lower in Candida in kidneys, eight-fold in liver and 11-fold in spleen was determined in the businesses of animals publish-dealt with the plant extract. These findings had been also showed by using Histopathological examination of the respective organs. [18]

Antioxidant activity: Treating broiler chicks with black seeds for 6 weeks avoided the liver from oxidative strain with the aid of growing the activities of enzymes which includes myeloperoxidase, glutathione-S-transferase, catalase, adenosine deaminase, myeloperoxidase and by way of lowering hepatic lipid peroxidation. [19]

Antidiabetic activity: Significant hypoglycaemic interest turned into pronounced. Antihyperglycemic outcomes of Nigella seed extract are attributed to a combination of the rapeutically applicable insulinotropic and insulin-like houses. [20]

Anti-inflammatory and analgesic activity: The chronic inflammatory disorders, allergies and arthritis involve a ramification of inflammatory mediators and pathways. Nigella fixed oil and thymoquinone have been discovered to inhibit membrane lipid peroxidation and eicosanoid generation in leucocytes, substantially decreased rat paw oedema and granulomapouch weight. Nigellonein low awareness is powerful in inhibiting the histamine release from the mast cells, which supports an antiasth maticrole for theplant. [21]

Immunomodulatory activity: The capacity immunomodulatory results of Nigella have been investigated in mild of splenocyte proliferation, macrophage function, and NK anti-tumor pastime the use of BLAB/c and C57/BL6 primary cells. Finally, experimental proof suggests that the aqueous extract of N. Sativa appreciably complements NK cytotoxic activity in opposition to YAC-1 tumor cells, suggesting that the documented anti-tumor results of N. Sativa may be, at least in component, attributed to its potential to function a stimulant of NK anti-tumor interest. It become expected that N. Sativa substances can be employed as powerful healing dealers inside the regulation of various immune reactions implicated in numerous conditions and illnesses together with most cancers [22].

III. Conclusion

Conclusion Ajwain seed possesses stimulant, antispasmodic and carminative residences and is used traditionally as an essential remedial agent for flatulence, atonic dyspepsia, diarrhea, abdominal tumors, abdominal pains, piles, and bronchial issues, lack of urge for food, galactogogue, bronchial asthma and amenorrhea. Medicinally, it's been validated to possess numerous pharmacological sports like antifungal, antioxidant, antimicrobial, antinociceptive, cytotoxic, hypolipidemic, antihypertensive, antispasmodic, bronchodilating movements, antilithiasis, diuretic, abortifacient, antitussive, nematicidal, anthelmintic and antifilarial. Further, research monitor the presence of numerous phytochemical materials specifically carbohydrates, glycosides, saponins, phenolic compounds, unstable oil (thymol, γ-terpinene, para-cymene and α and β-pinene), protein, fats, fiber and mineral remember containing calcium, phosphorous, iron and nicotinic acid. These research display that Trachyspermum ammi is a source of medicinally energetic compounds and feature various pharmacological consequences; consequently, it's far encouraging to locate its new therapeutic uses.

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Varpha-K Prototype, the Evolution of Anticoagulant Treatment with More Safety and Efficiency: Will this be the End of Bleeding?

By Cassius Souza, Sthéfany Estellet, Vitória Ferreira, Jonas Sardinha, Karina Delecrode, Blenda Graça, Leonardo dos Santos Pereira, Grazielly Ribeiro Viana, Karen Ritt, Gabriela Marques, Higor Franceschi Mota, Luciano Rapagña & Maria Sandra Ramos Queiroz

Abstract- Pharmaceutical chemistry seeks to discover and develop new chemical molecules that can be useful as medicines, in addition to the possibility of improvement in some molecular physicochemical aspect. These modifications cannot alter the pharmacophoric group, which is the chemical functional group responsible for the therapeutic effects (KOROLKOVAS 1982). Thus, our objective was to present the physicochemical aspects related to the association of vitamin K with warfarin molecule (VAR), in order to develop the Varfa-K Prototype, a new drug that has fewer toxic effects, mainly reducing bleeding events caused by VAR. Therefore, we used the methodology of molecular association and insertion of bulky groups. By adding a molecule (vitamin K) to the parent drug and its own antidote against bleeding.

Keywords: *drug, disposal, pharmaceutical chemical residues, environment.*

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Cassius Souza [α], Sthéfany Estellet [σ], Vitória Ferreira [ρ], Jonas Sardinha [ω], Karina Delecrode [¥], Blenda Graça [§], Leonardo dos Santos Pereira [χ], Grazielly Ribeiro Viana [ν], Karen Ritt [Θ], Gabriela Marques [ζ], Higor Franceschi Mota [£], Luciano Rapagña [€] & Maria Sandra Ramos Queiroz [₣]

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Keywords: drug, disposal, pharmaceutical chemical residues, environment.

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I. Introduction

Improving the quality of life has been a human purpose since the beginning of humanity. Thus, the cure and relief of diseases is an incessant search, for which the chemical synthesis of new medicines has contributed to the genesis of new drugs. Pharmaceutical chemistry seeks to discover and develop new chemical molecules that can be useful as medicines, in addition to the possibility of improvement in some molecular physicochemical aspect. One of the most efficient ways to create new bioactive molecules is to use molecules that already exist on the pharmaceutical market, for example, by hybridizing pharmacophoric groups (KOROLKOVAS 1982). All of this facilitates the creation of new drugs and the need for drugs that act in the treatment of bleeding, especially during surgical periods, where these clinical manifestations become much more common and aggressive. These modifications cannot alter the pharmacophoric group, which is the chemical functional group responsible for the therapeutic effects (KOROLKOVAS 1982). Molecular association is a method used for molecular improvement, where a different molecule of the original drug is added to increase the efficiency of the prototype. Thus, our objective was to present the physicochemical aspects related to the association of vitamin K with warfarin molecule (VAR), in order to develop the Varfa-K Prototype, a new drug that has fewer toxic effects, mainly reducing bleeding events caused by VAR.

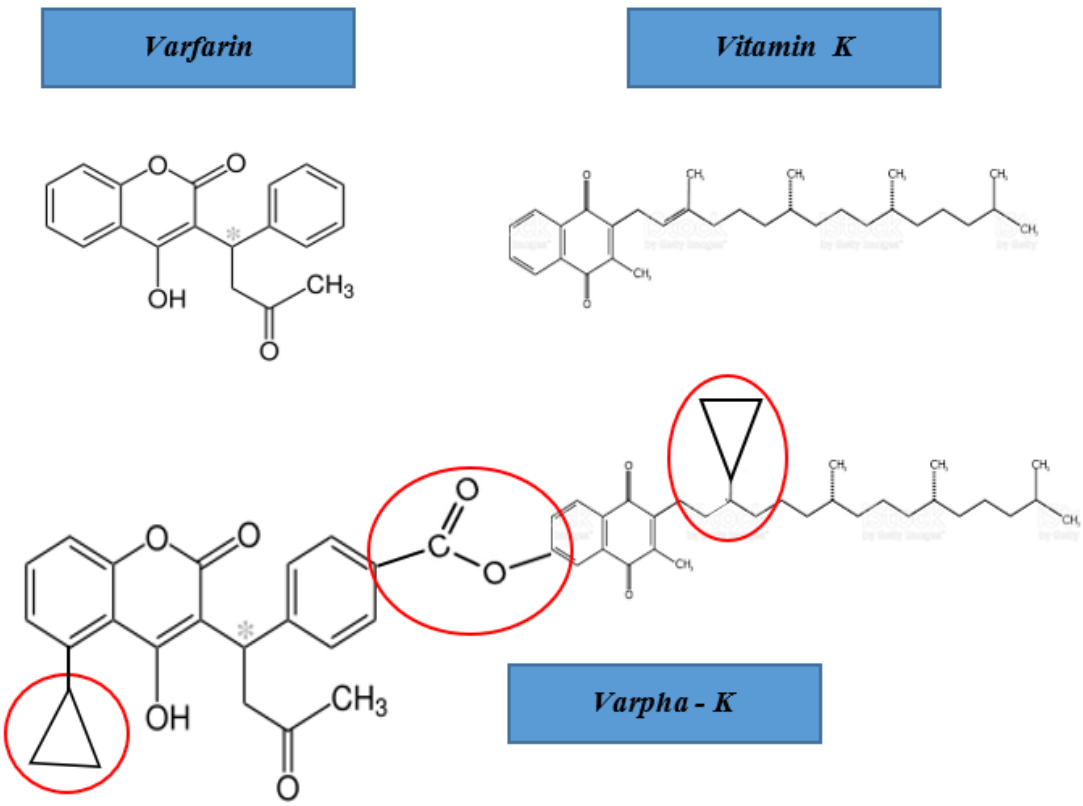
II. Methodology

Initially, a small bibliographic review was carried out using scientific outlets on Scielo and Pubmed, in addition, we established filters by publication time (5 years) and Portuguese or English language. Additionally, er also carry one used the methodology of

molecular association and insertion of bulky groups. By adding a molecule (vitamin K) to the parent drug and its own antidote against bleeding. The presence of the ester now will determine the decrease in metabolization of both molecules, since they will be separated by the action of the esterase in the biotransformation processes carried out in the liver.

III. Results and Discussion

The results with the development of this new molecular academic prototype varpha-K (figure 1) bring great expectations for patients who suffer from intense bleeding and that, depending on the condition, health can even lead to death. This new molecular architecture with the addition of the vitamin K molecule and the insertion of these bulky groups indicates that there will be a favor for the reduction of toxic clinical manifestations associated with the traditional VAR molecule, such as the dreaded tissue bleeding, since vitamin K has anti-hemorrhagic action, in addition to promoting increased bioavailability thanks to the presence of these bulky groups, allowing the new drug to take longer to be metabolized by Cytochrome P450 enzymes.



Legends: Image of warfarin and vitamin K molecules, as well as that of the academic prototype Varpha-K

IV. Conclusion

Our studies are just academic hypotheses, but with great therapeutic expectations, since, to this day, there are few studies for improving medication and especially for the toxic effects of warfarin.

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Biofield Energy - Health Effects of Solar Flares by Seema Bhattessa MR Pharms

By Seema Bhattessa

Abstract- Space weather is the term used to describe events, or storms, in space that affect humans. These events, including solar flares, coronal mass ejections, and their associated electromagnetic effects, are primarily attributed to magnetic shifts occurring on the Sun. These events intensify during solar maximum, the phase of the 11-year solar cycle leading up to the Sun's polar shift, during which the Sun's magnetic north and south poles switch. The resulting geomagnetic storms reach and affect Earth's atmosphere, with impacts on electrical systems, including power infrastructure, communications systems, and biological systems. Epidemiological studies show correlations between solar activity and increased severity of neurologic symptoms, increased rates of cardiovascular events, and behavioural, immune, and other effects. Currently, the Sun is approaching solar maximum, which will peak in 2025, bringing an increased frequency and intensity of geomagnetic storms.

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I. Storms in Space

Space weather, the term used to describe events, or storms, in space that affect humans, is a subset of astrophysics that originated in the early 19th century. A naturalist at that time observed magnetic needles oscillating concurrently with the appearance of auroras, emissions of red, green, purple, and blue visible light that occurs when particles from a space storm collide with oxygen and nitrogen in Earth's upper atmosphere (Buzulukova & Tsurutani, 2022).

Normally, Earth is shielded from the effects of solar activity by a strong magnetic field called the magnetosphere, which protects our atmosphere by repelling charged particles carried towards Earth from space weather events. However, certain conditions, such as fluctuations in solar winds, can disrupt the magnetosphere and allow solar energy and mass to reach Earth's atmosphere, generating magnetic disturbances known as geomagnetic storms.

The largest geomagnetic storm ever recorded occurred in 1859 and is known as the Carrington event. This space storm affected the majority of the planet, with auroral effects that persisted for several days. Although public health records do not show any health effects, possibly due to limitations in the monitoring systems of the time, the geomagnetic currents induced by the Carrington storm disrupted telegraph systems, with some stations experiencing fires due to induced electrical currents (Muller, 2014).

The majority of space weather events arise from two main categories of solar activity: active regions and coronal holes.

Active regions are areas of intense magnetic activity. They mainly occur during the phase in the solar cycle leading up to the polar shift. Active regions give rise to major solar events such as solar flares and coronal mass ejections.

- Solar flares are sudden releases of energy resulting from reconfiguration and reconnection of the magnetic fields within the Sun's photosphere, the innermost layer of the Sun's atmosphere and the layer that emits the most energy. Solar flares are the most powerful explosions in our solar system, equivalent to the energy of billions of hydrogen bombs. Photons released in a solar flare travel at close to the speed of light and range in frequency from ultraviolet (UV) to X-rays.
- Coronal mass ejections are giant magnetised plasma structures (superheated, high-energy ions) containing millions of tonnes of matter that are ejected from the outermost layer of the Sun. They occur when the Sun's magnetic fields reconnect. As a coronal mass ejection moves away from the Sun it expands and becomes a giant magnetic cloud, some reaching millions of miles in width, with high magnetic field intensities capable of causing geomagnetic storms on Earth.

Coronal holes are regions in the Sun that have magnetic fields in which the field lines are attached to the Sun but are pulled outward into the heliosphere by solar winds, referred to as open magnetic fields. These contrast with closed magnetic fields, which form closed loops that remain within the solar corona(Fisk & Schwadron, 2001). Open magnetic fields interact with closed magnetic fields, generating solar winds with very high speeds of up to 800 km/s. Magnetic field fluctuations in these wind streams are known to cause geomagnetic activity on Earth.

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II. Helio Biology: The Study of Space Weather

Various types of space weather, including geomagnetic storms, solar flares, and cosmic rays—high-speed atomic particles that travel through space at close to the speed of light—are thought to have detrimental effects on human health. Cosmic rays originate both from the Sun, known as solar energetic particles, and outside of the solar system, known as galactic cosmic rays. Solar cosmic rays can increase by 100% during strong solar flares(J.-C. David a, 2019).

Helio biology, a recently created branch of science, studies the health impacts of solar activity. In order to determine the potential effects of solar flares and other types of space weather on human health, data is collected on the temporal associations between the occurrence of solar events and medical data. Relevant data include rates of hospital admissions and emergency room visits, physiological parameters, such as changes in heart rate variability and blood pressure, alterations or effects on microcirculation, and effects on reaction time, and data from laboratory tests and tissue samples (Abdullrahman HM, 2020).

A number of Helio biology studies have reported pronounced effects on cardiovascular, neurologic, and mental health. These systems of the body are particularly reliant on microcurrents of electricity to function (Abdullrahman HM, 2020). Evidence for immune and other health effects has been documented as well.

The adverse health effects of space weather have been presumed to be more pronounced in those with already compromised health. However, the electromagnetic energy arising from solar activity may be compounded under certain conditions, such as emotional stress, and in certain environments, such as being inside the metal structure of an automobile, with effects estimated to be as much as 30% greater under these conditions (Zakharov & Tyrnov, 2001).

a) *Cardiovascular Effects*

Various consequences related to cardiovascular health have been noted in conjunction with periods of high solar activity, including decreased heart rate variability, increased blood viscosity, increased blood pressure, and increased rates of myocardial infarctions (Abdullrahman HM, 2020). Animal studies have demonstrated the degradation of cardiomyocytes in rabbits during severe geomagnetic storms (Vencloviene et al., 2014).

A large U.S. study analysing 44.2 million deaths over a 30-year period found evidence for the effects of geomagnetic disturbances on deaths from cardiovascular diseases, myocardial infarction, and stroke(Zilli Vieira et al., 2019). Moreover, some studies have noted increases in sudden cardiac deaths and fatal myocardial infarctions on days when the levels of geomagnetic activity are either at their highest or lowest, and on days with high cosmic ray activity (Mavromichalaki et al., 2021).

A study that evaluated acute coronary event risks in relation to geomagnetic storms, solar energetic particles, and solar flares found from 1.6 to 2.8 times increased risk for cardiovascular death, coronary artery bypass graft, and acute coronary syndrome (Vencloviene et al., 2014). The most statistically significant increases in myocardial infarctions and strokes have been observed when geomagnetic disturbances occur in conjunction with rapid decreases in cosmic ray intensity following a coronal mass ejection, known as Forbush decreases (Mavromichalaki et al., 2021). These health events occur with greater frequency during the main phase of the decrease.

There is evidence that some cardiovascular effects are more pronounced in women. A study in Bulgaria found statistically significant increases in mean arterial systolic and diastolic blood pressure in relation to fluctuations in geomagnetic indices. Greater sensitivity to these effects was observed among women compared to men (Mavromichalaki et al., 2021).

A study encompassing more than 16,000 myocardial infarction patients in Lithuania over a 17-year period found a significant correlation between solar activity and geomagnetic indices and cosmic ray activity levels, also with a stronger correlation in women. And, in a study of stroke mortality related to sunspots—areas of reduced temperatures on the Sun's surface due to decreased convection from magnetic flux—54% were women (Mavromichalaki et al., 2021).

It has also been noted that those taking hypertension medications have increased cardiovascular responses, particularly with regard to arterial, pulse, and systolic blood pressures, during severe geomagnetic storms (Dimitrova, 2006).

b) *Neurologic Effects*

Various effects of space weather on brain and behavioural function have been documented. Neurologic changes include increased frequency of epileptic seizures, changes in breathing patterns, and increased flareups of neurological conditions (Papathanasopoulos et al., 2016). Changes in mental and behavioural health include increased rates of depression and mental illness, significantly increased numbers of suicide attempts, homicides, and traffic accidents, and exacerbation of symptoms related to schizophrenia and Alzheimer's disease (Abdullrahman HM, 2020).

A study conducted in Karkhiv, Ukraine, found that effects were most pronounced at the later stages of a geomagnetic storm, during the recovery phase of the storm (Zakharov & Tyrnov, 2001). The researchers recorded emergency room visits, psychiatric

hospitalisations, and pediatric psychoneurology appointments during ten 27-day solar rotations in 1993, which included one period of increased solar flare activity and three geomagnetic storms. An increase also occurred during the geomagnetic storm recovery period.

Neurological impairment from space weather events has also been associated with increased numbers of train and car accidents related to human error, with some studies finding a positive correlation with cosmic rays and a negative correlation with solar activity, while others note the opposite. A study of the effects of geomagnetic activity on brain and psycho-emotional health in female volunteers found that stress levels and the ability to focus were impacted (Mavromichalaki et al., 2021).

Increased hospital admission rates have been noted in multiple sclerosis patients due to acute relapses correlating temporally with intense geomagnetic storms (Papathanasopoulos et al., 2016). Data collected over an 11-year period from a hospital in Greece showed the increase in admissions occurred shortly after these storms, with a secondary, larger increase occurring 7–8 months later.

A theory has been put forth linking space weather as a potential contributing cause of multiple sclerosis. It is thought that neuromelanin, a neuroprotective molecule in the brain similar in structure to melanin, becomes oxidatively charged in response to space weather events, making it unable to eliminate reactive oxygen species in the brain and thereby promoting demyelination. This theory is supported by the global distribution of multiple sclerosis, with prevalence lowest at the equator and increasing rapidly in north and south directions until peaking at about 60° north and south, corresponding more closely to geomagnetic than geographic latitude (Papathanasopoulos et al., 2016).

c) Metabolic, Immune, and Genetic Effects

The electromagnetic fluctuations that we experience in Earth's atmosphere from space weather events cause metabolic stress to cells as they attempt to maintain homeostasis. This metabolic stress can lead to increased inflammatory cytokine levels, bringing its effects on the immune system and its ability to function effectively in the context of space weather events into consideration (Davis & Lowell, 2004).

As an example, one study found a significant increase in H. pylori antigens in saliva and feces samples of volunteers after a powerful solar flare that occurred in 2017, indicating a decreased ability of the immune system to suppress the infection (YA Belaya, 2019). Interestingly, however, one study found a negative correlation with immune symptoms, in which a decrease in symptoms was observed in patients with lupus erythematosus, a rheumatological autoimmune disease, during increases in geomagnetic activity (Stojan et al., 2021).

Genetic effects have been reported in a Russian study that found a destabilizing effect of solar flare activity on DNA and other components of cell nuclei (Kalaev, 2023). Cheek swab samples showed changes to the nuclei of the cells, including the appearance of notches, micronuclei, protrusions from the nuclei described as having a "broken egg" or "tongue-like" appearance, and other abnormalities.

Reproductive effects include increased incidence of Down syndrome and other congenital and chromosomal anomalies, as well as premature births (Abdullrahman HM, 2020).

III. Proposed Mechanisms

Several mechanisms have been proposed to explain the health effects of solar flares and other types of space weather. One theory considers that biological systems have evolved within Earth's fluctuating electromagnetic fields, and the frequencies emitted by geomagnetic storms may be close to and resonant with the characteristic frequencies of certain internal organs, thereby affecting them on a cellular level (Abdullrahman HM, 2020). Resonant electromagnetic waves that occur between the Earth and the ionosphere, known as Schumann resonances, have a similar frequency to human alpha brainwaves (Vencloviene et al., 2014). And certain geomagnetic micro pulsations correlate with the frequency of human heart rhythm, while others are similar to the oscillation frequency of endothelium, which can increase the risk of coronary events (Vencloviene et al., 2014).

Another prevalent theory involves the suppression of melatonin from the pineal gland. The pineal gland contains high levels of a permanently magnetised form of iron oxide called magnetite, making it relatively more susceptible than other organs or tissues to changes in magnetic fields (Gilder et al., 2018). Since melatonin regulates the body's circadian and biological rhythms, decreased levels are thought to impair mental health, making individuals more susceptible to depression (Abdullrahman HM, 2020), and cardiovascular health, with disturbances leading to heart arrhythmias, heart failure, and other cardiovascular effects (Vencloviene et al., 2014).

Also involved in circadian rhythms is a magnetically sensitive structure in the retina known as the cryptochrome system. Physiologic stress from geomagnetic fluctuations may affect the hypothalamic–pituitary–adrenal stress response system via disruptive and disorienting effects on the cryptochrome system (Close, 2012).

Space weather events can have electrically destabilising effects on cells. Neurologic damage from geomagnetic field fluctuations may arise from inhibitory

effects on the sodium–potassium pump, the mechanism by which cells maintain electric charge across their outer membrane. In nerve cells, this destabilising effect may cause excessive influx of calcium, leading to nerve cell degeneration or death and symptom flareups in neurological conditions (Papathanasopoulos et al., 2016). In heart cells, on days with high levels of cosmic ray activity, the resulting electrical instability has been found to damage cardiomyocites in patients with ischemic cardiopathy (Stoupel et al., 2008).

With regard to genetic effects, X-rays and UV radiation emitted during geomagnetic events have potentially damaging effects on myocardium (Vencloviene et al., 2014). Geomagnetic activity has been purported to influence the expression of NF-kappaB, which activates transcription of DNA, controls the production of immune-signalling cytokine molecules, and is important for overall cell survival (Papathanasopoulos et al., 2016).

a) Mitigating the Health Impacts of Space Weather

The electromagnetic fields induced by solar flares and other space weather events constitute a form of low-level radiation exposure of both non-ionising and ionising types. Various nutrients have been found to have protective effects, reduce cellular damage, and preserve cellular function.

b) Antioxidants

Antioxidants such as vitamin E, vitamin C, selenium, beta-carotene, N-acetyl cysteine, and alpha-lipoic acid have all been demonstrated to protect against radiation-induced cellular changes in human studies. Moreover, the benefits of antioxidants are greater when used in combination than individually (Prasad, 2005).

IV. Medicinal Herbs

A number of medicinal herbs contain polyphenol antioxidant compounds that reduce inflammation, directly protect cells against radiation, and promote the production of powerful antioxidant enzymes such as superoxide disumutase, glutathione peroxidase, and glutathione S-transferases. Examples include ginseng, reishi mushroom, olive leaf, hops, yarrow, and alliums (Mohamad Ali Dayani, 2019). Many medicinal plants have been found to mitigate neuronal damage in the brain by virtue of their antioxidant and anti-inflammatory effects, including black cohosh, wormwood, cinnamon, saffron, turmeric, salvia, ginseng, rosemary, and others(Keshavarzi et al., 2019).

a) Essential Minerals

Calcium and magnesium are required for nerve conduction and muscle contraction and relaxation. Deficiencies or the presence of these minerals in suboptimal proportions may increase the risk of detrimental effects from the added stress of geomagnetic events. Certain nutrient minerals have also been shown to have radio-protective effects. Copper, iron, manganese, and zinc all serve roles in protecting cells against radiation injury as components of antioxidant enzyme complexes and metallochelates (Sorenson et al., 1995). Minerals are depleted in modern soils and therefore, in diets; supplementing can help ensure optimal levels.

b) Chlorophyl

Chlorophyl offers numerous health benefits that can help reduce the harmful effects of space weather events. Chlorophyl reduces oxidative stress, helping the body safely metabolize harmful chemicals, and it upregulates genes responsible for inhibiting cancer (Hayes & Ferruzzi, 2020). Its structure is similar to that of human hemoglobin, with magnesium in place of iron as the oxygen-binding portion of the molecule. Concentrated sources of chlorophyl are found in barley grass and Chlorella, a genus of algae.

c) Dietary Choices

Diet may play a significant role in protecting against metabolic stresses from geomagnetic events. Cruciferous vegetables contain the compound sulphoraphane, which reduces oxidative stress and inflammation(Ruhee & Suzuki, 2020). Fermented foods offer neuroprotective benefits by maintaining a healthy gut microbiome, which protects the gut mucosal barrier and prevents intestinal permeability, which can lead to increased production of neurotoxic compounds by various pathogenic microbes, and an increased neuroinflammatory response in the brain(Porras-García et al., 2023). Foods high in soluble fiber promote a healthy gut microbiome by providing a source of energy for beneficial bacteria, and both soluble and insoluble fiber speeds the elimination of waste, thereby speeding the elimination of waste products generated by the increased metabolic stress induced by geomagnetic events. Additionally, essential fatty acids have important anti-inflammatory and immune-regulating effects(Newell et al., 2021).

V. Conclusion

As the Sun's magnetic field activity reaches the peak of its 11-year cycle, major electromagnetic events known as space storms occur with increasing frequency and intensity. These storms impact Earth's atmosphere, with disruptive effects on electrical infrastructure and communications systems as well as on biological systems, which experience a form of metabolic stress during these events. Epidemiological studies show temporal correlations between solar activity and neurological, cardiovascular, behavioral, and other aspects of health. Certain nutrients and plant compounds are capable of mitigating radiation-induced cellular and metabolic damage. Awareness of solar

cycles, particularly the peak solar activity phase, and utilizing available options for minimizing potential health effects may help reduce the public health burden associated with these events.

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Effect of Ozoniotherapy in the Treatment of Necrosis after Hair Transplantation: Case Report

By Luisa Melo Lucas, Anayene Craveiro Mendes & Jorge Temer Merhi

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Abstract- *Introduction:* Nowadays, ozone plays an important role in wound healing and tissue repair as a therapy and as antimicrobial, bactericidal and fungicidal agent. It attains recognition in hair transplantation as a treatment for necrosis due to hypoxic-ischemic local syndrome. *Objective:* to demonstrate the therapeutic evolution of ischemia in a hair transplant after ozone therapy sessions. *Methods:* The patient was evaluation and gave consent to photographic records. He went through 30 topic applications of ozonated oil, with a 10 drop dosage daily; 12 bag ozone sessions for 10 minutes; besides subcutaneous applications, with 30% ozone concentration and a very small gas volume (1-2 ml) with 30G needle. *Results:* Evolution of the case was registered with images and tissue coloring and changes evidenced. *Conclusion:* It is clear that ozone therapy made wound healing and tissue repair faster, since there was an increase of epithelial cells and neoangiogenesis due to therapy, resulting in almost complete repair of the patient's hair transplant at the end of the sessions.

Keywords: *ozone therapy, hair transplant, hair treatment.*

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Abstract- ***Introduction:*** **Nowadays, ozone plays an important role in wound healing and tissue repair as a therapy and as antimicrobial, bactericidal and fungicidal agent. It attains recognition in hair transplantation as a treatment for necrosis due to hypoxic-ischemic local syndrome.** ***Objective:*** **to demonstrate the therapeutic evolution of ischemia in a hair transplant after ozone therapy sessions.** ***Methods:*** **The patient was evaluation and gave consent to photographic records. He went through 30 topic applications of ozonated oil, with a 10 drop dosage daily; 12 bag ozone sessions for 10 minutes; besides subcutaneous applications, with 30% ozone concentration and a very small gas volume (1-2 ml) with 30G needle.** ***Results:*** **Evolution of the case was registered with images and tissue coloring and changes evidenced.** ***Conclusion:*** **It is clear that ozone therapy made wound healing and tissue repair faster, since there was an increase of epithelial cells and neoangiogenesis due to therapy, resulting in almost complete repair of the patient's hair transplant at the end of the sessions.**

Keywords: ozone therapy, hair transplant, hair treatment.

I. Introduction

Hair transplant has been changing many individuals' reality that face alopecia, a disease consisting of head or body hair loss. Unlike several other transplants, hair transplant is in the spotlight because of its peculiarity in using follicles of the same donor who is supposed to receive them in a less invasive way. In spite of the many techniques available, all of them require patient evaluation including: patient history, age, previous medical evaluations. Diagnosing the type of alopecia is mandatory. Whenever the patient has the conditions for the procedure, it is unusual the occurrence of resulting complications. However, as highlighted by Zito and Raggio in Statpearls, "Potential complications include: edema (5%), bleeding (0.5%), folliculitis, infection (less than 1% of patients)". Being the necrosis of the receiving area due to excess density in the area or another possible cause present.

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Hypoxic-ischemic local syndrome, which can evolve to tissue necrosis, develops from low blood perfusion in tissues and decrease in oxygen because of several etiologies, such as abuse of anesthetics and vasoconstrictors and excess of FUs in an area. The appropriate level of oxygen in tissues is fundamental so that cells keep their aerobic metabolism and vital functions. When the perfusion pressure is not enough to keep the minimal oxygen level, aerobic metabolism shifts to anaerobic with resulting organic dysfunctions. Therefore, treatment is an issue of diagnosing the primary cause and should be initiated at the ischemic lesion spot so as to start revacularization.

Necrosis takes place whenever a cell is exposed to extreme environmental conditions, adverse and excessive stimuli, or in face of deleterious mutations codified in its genetic material. Cell necrotic death occurs as a response to severe physiological conditions, including hypoxia, ischemia, toxin exposition, anesthetics, reactive metabolites of oxygen and nutrient deprivation. In cases of ischemic necrosis, nuclear alterations of cytoplasms portray a clotted blood appearance: acidophilus, granular and hardened. There is loss of tissue structure and the area becomes whitish, bulged and hyperemic. Among microscopic aspects there is increase in acidophilus, a granular appearance and formation of amorphous masses as a result of membrane rupture and mixture of autolyzed material.

Ozone therapy, considered an alternative therapy, with excellent results and ease of application, is in evidence in many countries. It was first acknowledged in 1839, by german chemist Christina Friedrich, and in 1896, by Nikola Tesla, who patented the first ozone generator, in the US, used during the First World War to treat gas gangrene, which treatment is still in use.

Ozone therapy is a bio-oxidative therapy based on a gasified mixture of oxygen and medical ozone, whose therapeutic effects include mainly the improvement of metabolism and the oxygenation of peripheral tissues, as a consequence of increased erythrocyte flexibility, allowing for a better flow inside capillaries and assuring a larger supply of oxygen in the tissues. This process facilitates epithelial repair and growth and inhibits bacterial and fungicidal development.

In hair transplantation, despite technological advancements, ozone therapy application to treat ischemic necrosis is unknown or barely known, as

shown by the reduced number of research papers and therapeutic approaches which might be a guidance for professionals in the field. Thus, the current study aimed at making the causes and effects of ozone therapy more clear whenever it is aptly applied to treat and prevent ischemia and necrosis in hair transplantation procedures.

II. Materials and Methods

This is a longitudinal descriptive and interventionist study with convenience, consecutive, non-probabilistic sampling. The patient underwent a hair transplant procedure in May, 2022, in an unknown doctor's office and was referred to Dr. Anayene Craveiro, at Belcorp Institute, after first signs of ischemic necrosis.

The recommendations of the Madrid Declaration on Ozone Therapy were considered to evaluate the appropriate doses for the corresponding mechanism of action. First, there are three basic principles: (1) not to do harm; (2) stagger the dose; (3) apply the necessary concentration.

Treatment was started with initial evaluation and recognition of the ischemic necrotic area, with mediated intervention. Lesion characteristics were evaluated on the grounds of photographic records facilitating the patient's therapeutic evolution follow-up.

The Oxy device, manufactured by Tonederm®, licensed by the Brazilian Health Regulatory Agency (ANVISA), was employed in the treatment. This device turns medical oxygen into ozone gas through corona discharge. Topical treatment with gas, and a plastic transparent bag manufactured with ozone resistant material, consists of applying an elastic band with sealed edges to the skin.

III. Case Outline

A 40-year-old white male patient, with no pre-existing diseases, underwent the hair transplantation procedure in May, 3rd 2022, with 4,600 follicle units.

The patient - himself a doctor - was referred to Dr. Craveiro Mendes in the same week following his noticing of an ischemic area. His exams showed no other symptoms, nor were there any complaints of allergic reactions. On inspection, the lesion showed well defined edges adherent to wound bed with small fibrin clots, wound bed with granular tissue, adjacent skin edema, peeling skin around the tissue lesion and absence of exudate and odor.

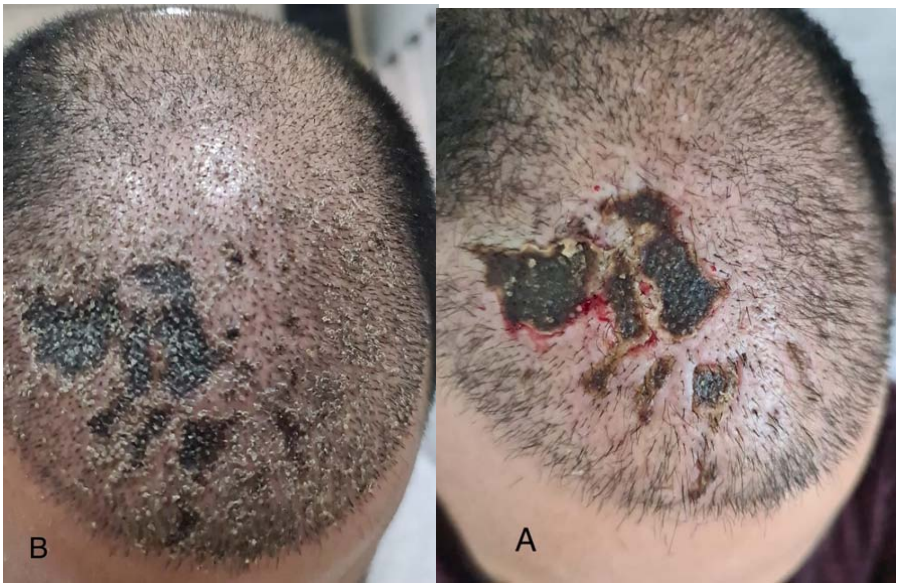
The patient was submitted to 30 ozonized oil topical applications, 10 drops a day, and twelve 30 % ozone sessions with a bag, once a week, for 10 minutes, besides subcutaneous 30% concentration ozone applications with a small gas volume (1-2 ml) through 30G needle. Ozone therapy was conducted after local hygienization with no dressing following the application.

The patient, who unexpectedly faced complications after pursuing hair transplant for high self-esteem, was also provided with psychological care for better acceptance of ozone therapy results.

IV. Results and Discussion

Photographic images demonstrate the progress between the first and last ozone therapy applications. There was local neovascularization and wound healing with progressive reduction of the necrotic area. It is possible to observe at first hand the increased blood supply, vessel permeability and vasodilation, which showed a better coloring appearance since the first session. Granular tissue was found in the first session with endothelial and fibroblast proliferation, which are mesenchymal differentiated cells spreading on the lesion surface. On the first days, angiogenesis first stages were observed with a bulged and whitish region surrounded by a red halo. On the last day it was possible to see a better wound bed and epithelial tissue growth, that is, new skin growing out of the lesion edges in face of a concentration process of the marginal wound walls, under the action of activated fibroblasts, making epithelization possible. It exhibits a shiny rose coloring related to mature collagen.

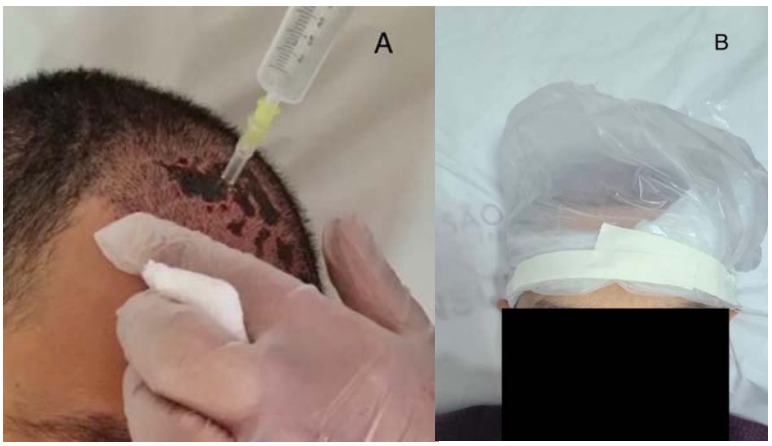
According to the photographic records before and after the three sessions (Picture 1), there was improvement of tissue healing, decreasing bulging, better local blood supply, and recovery of the whitish appearance. In addition, there was growth of granular tissue due to collagen activity, elastin and reticular fibers in an attempt to tissue repair. This phase produces the increase of inflammatory cells, growth factors, vasodilation and presence of permeability.



Source: Author

Picture 1: Before (B) and after (A) of Ozone Therapy Application

Along the application sessions, there was significant improvement (Picture 2). Necrotic tissue started debriding and granular tissue formation took place, with faster neovascularization and local epithelialization. Studies have demonstrated that ozone oil can promote wound healing through PI3K/Akt/mTOR signaling. Mechanically, it is possible to verify that ozone oil can activate fibroblasts and promote their migration. Besides that it can extend the mesenchymal epithelial transition (MET) process.



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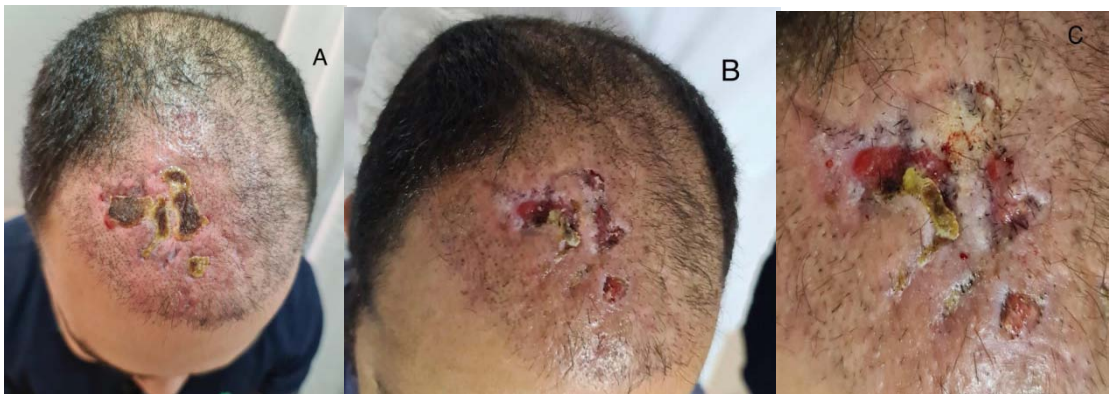
Picture 2: Application of Subcutaneous Ozone (A) and Bag Therapy (B)

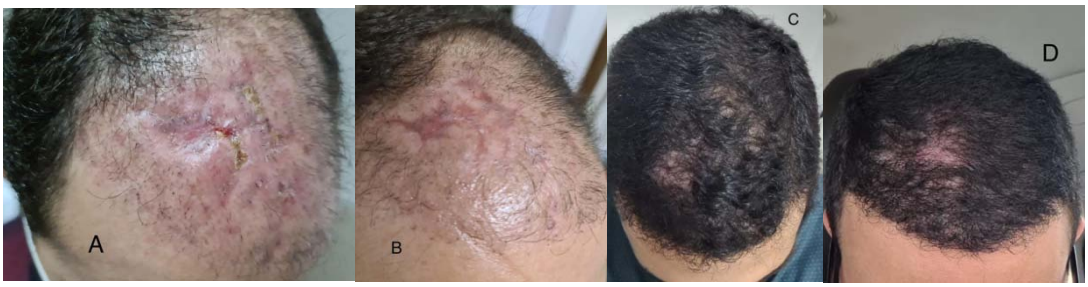
The analysis of therapeutic evolution after 5 sessions of ozone therapy (Picture 3) makes clear the expansion of mesenchymal cells, fibroblasts, on the wound surface, which is related to internal vessel growth and formation of conjunctive tissue. From this moment, concentration of lesion edges takes place, facilitating epithelization.



Source: Author

Picture 3: Evidence of mesenchymal cell growth surrounding de lesion (A); Concetration of edges for epithelization in (B) and (C)

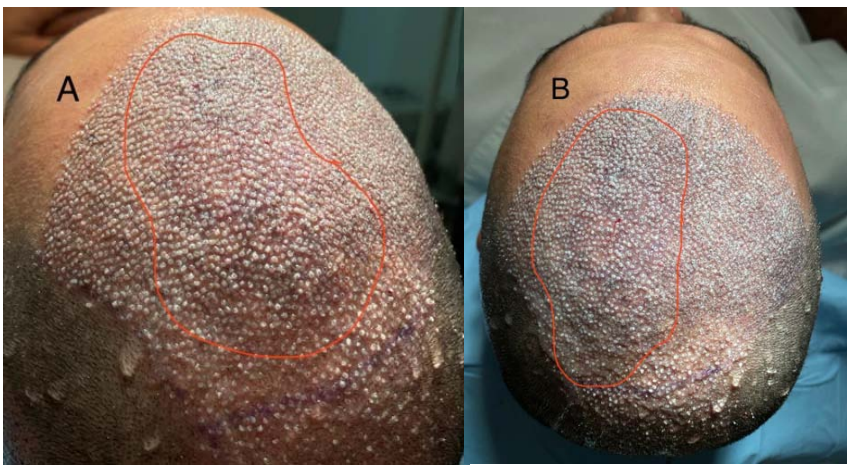
Gradually after concluding ozone therapy sessions, remodeling phase starts (Picture 4) leading to reduction of cell activity and blood vessels, Then, maturation and increased local resistance ensues.



Source: Author

Picture 4: Tissue with reddish coloring indicating blood flow and mature collagen (A) and (B). There was hair growth of some follicles implanted in the area, which demonstrates recovery from the hair transplant through ozone therapy (C) and (D)

For data collection, pictures after hair transplantation procedure (Picture 5). High density and dark coloring areas due to possible ischemia are visible.



Source: Author

Picture 5: Hair transplant with high approximation of FUs in (A) and (B) showing spots with immediate reduction of blood flow

There is limited evidence on the direct use of ozone therapy in hair transplantation, but it is successful in several other treatments and it presents a therapeutic challenge. There are several therapies for dermal treatment but their adverse effects hamper their application. However, as previously observed, ozone therapy, despite being a simple molecule, holds an

efficient approach to fight microorganisms and promote healing capacity.

V. Conclusion

The current study demonstrated the use of ozone for ischemic tissue treatment. Eventual therapeutic outcomes were positive as healing evolution was attested as a result of improved blood flow and re-epithelialization of damaged tissue.

Despite being an innovative procedure, hair transplantation does not exclude the possibility of necrosis, which highlights the importance of the availability of tools to cope with unexpected situations. Healing is a complex process and demands immediate intervention in face of its occurrence.

This case report is free of any conflict of interest and aims at supporting study and learning initiatives by professionals addressing similar cases in their professional settings.

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Development of a Urinary Screening Method, a Urinary Quantitative Confirmatory Method and Metabolic Profiling of the Semi-Synthetic Cannabinoid Hexahydrocannabinol in Humans Using UPLC-MS-QToF and UHPLC-MS/MS

By K. Kemenes, E. Hidvégi & G. Süvegh

Abstract- Hexahydrocannabinol (HHC) is a semi-synthetic drug structurally related to natural cannabinoids, manufactured from hemp-extracts. By cyclization and catalytic hydrogenation, cannabidiol (CBD) is converted into a mixture of (9R)-HHC and (9S)-HHC. The (9R)-HHC epimer has THC-like psychoactive effects.

HHC was first reported to EMCDDA in August 2022. By April 2023, 20 EU Member States have detected the substance. Human metabolism studies and analytical methods for investigating HHC metabolites in human urine are lacking.

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This study aims (I) to present an analytical method for screening HHC metabolites in human urine; (II) to investigate the Phase I HHC metabolites by performing *in vitro* and *in vivo* metabolite identification studies of human liver microsome (pHLM), S9 fraction (pS9) and urine samples; (III) and to present a quantitative confirmatory method optimised by the investigation of authentic urine samples.

1. The screening method was developed by the MRM-optimisation of a glucuronic acid conjugated carboxy-HHC-metabolite on a Shimadzu LCMS-8040 triplequadrupole massspectrometer. It was based on the identification of the metabolite in a (9R)-HHC self-administration urine sample by a Waters Xevo G2-XS QToF system.
2. The authentic urine samples screened positive for this HHC-metabolite and confirmed positive for HHC parent compound were used for the metabolic profiling assay. Thirteen *in vivo* Phase I urinary metabolites of HHC epimers were tentatively and partially identified and confirmed by *in vitro* metabolites detected in pHLM and pS9 samples. Six oxidised metabolites, six carboxylated metabolites and one double-carboxylated metabolite were detected in eleven urine samples of HHC users. All putatively identified metabolites were more abundant in urine than the parent compound.
3. The development of the confirmatory method on a Shimadzu LCMS-8050 triplequadrupole mass spectrometer and the optimisation of the hydrolysis step were based on the quantitation of the commercially available reference standard, (9R)-11-COOH-HHC by investigating thirty-one positive authentic urine samples.

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I. Introduction

The recreational use of preparations of the flowering or fruiting top of *Cannabis sativa* has been internationally controlled since 1961 in Article 1 of the Single Convention on Narcotic Drugs (1). Despite all the legislative efforts, cannabis is the most popular and widely used recreational drug according to the 2022 World Drug Report of the United Nations Office on Drugs and Crime (2). However, to circumvent legislation, new compounds having cannabimimetic effects have emerged in the last decades. From the 2000s, different series of highly potent synthetic cannabinoids have been appearing and disappearing on the drug market. The abuse of these compounds have become widespread due to their low price, strong psychoactive effect and the lack of legal control, (3). The introduction of the 2018 Farm Billresulted a significant change in the composition of the cannabinoid market.It led to the overproduction of phytocannabinoids, like CBD, in the US.The overproduction of their precursor led to the rapid spread of newly emerging, uncontrolled, semi-synthetic cannabinoids, like Δ8-THC, HHC ortheir analogues (4).HHC was first reported to the European Monitoring of Drug and Drug Addiction (EMCDDA) in Denmark in August 2022 (5). By April 2023, 20 EU Member States have detected the substance. In April 2023, the EMCDDA released an extensive and detailed Technical Report on the rapidly emerging semi-synthetic cannabinoids (6).

Hexahydrocannabinol can be found naturally in trace amounts in *Cannabis sativa*. However, the large-scale production of HHC uses phytochemical precursors like CBD from hemp extracts. Hemp-derived cannabidiol (CBD) is converted into a mixture of (9R)-HHC and (9S)-HHC epimers by simple chemical transformations like cyclization and catalytic hydrogenation. There are three stereogenic carbon atoms in HHC (6a, 9, 10a), resulting theoretically eight

epimers, but only the (6aR, 9R, 10aR)-HHC and the (6aR, 9S, 10aR)-HHC isomers have appeared so far (7), scientific studies focus on these isomers. Pharmacological studies indicate that only the (9R)-HHC isomer has cannabimimetic psychoactive effects. This is explained bythe spatial position of the C-11 methyl group being equatorial, so it is located in the plane of the quasiplanar alicyclic system, just similar to the C-11 methyl group of Δ9-THC. The (9S)-HHC isomer has an axial C-11 methyl group pointing out of the plane of the quasiplanar alicyclic system, resulting in the loss of receptor binding affinity and the loss of psychoactive pharmacological effects (8).

A comprehensive review from Istvan Ujvárysummarises several UPLC-MS/MS, GC-MS, GC-MS/MS, TLC, IR, NMR methodologies for the structure identification of HHC in different matrices (4). Most of them are focused on the determination of the parent compounds. Baseline separation of the two epimers can be achieved with all techniques allowing quantitative analysis.

Forensic toxicology is highly based on urine analysis, it is essential to identify suitable urinary biomarkers of hexahydrocannabinol consumption and monitor them. Therefore, it is equally important to study the excretion profile, the metabolism and the pharmacology of these compounds. However human metabolism studies and analytical methods for investigating HHC metabolites in human urine are lacking or controversial. Due to the lack of adequate analytical methods to detect HHC in biological matrices, actual intoxication cases have not yet been reported, and the harmful potential of HHC was not described (9).

This study aims (I) to present an analytical method for screening HHC metabolites in human urine; (II) to investigate the Phase I HHC metabolites by performing *in vitro* and *in vivo* metabolite identification studies of human liver microsome (pHLM), S9 fraction (pS9) and urine samples; (III) and to present a quantitative confirmatory method optimized by the investigation of authentic urine samples.

1. The screening method was developed by optimizing a glucuronic acid conjugated carboxy-metabolite on a Shimadzu LCMS-8040 triplequadrupole mass spectrometer, based on a (9R)-HHC self-administration urine sample after the sample had been investigated and the metabolite had been tentatively identified on a Waters Xevo G2-XS QToF system. The QToF method had been transferred to the triple quadrupole system. After the optimized MRM-transitions of the glucuronic acid conjugated carboxy-metabolite were added to the routine LC-MS/MS screening method, the authentic urine samples screened positive for the HHC-metabolite by the LC-MS/MS analysis and confirmed positive for the HHC parent compound by the QToF analysis were collected and used for the metabolic profiling assay and the development of routine confirmatory analysis.
2. The analytical strategy for metabolic profiling was described in detail in our previous work (10).Based on the analysis of urine samples from users (n=11) as positive controls, blank urine samples as negative controls, pooled human liver microsome (pHLM), and pooled S9 fraction (pS9) samples *in vitro,* using a Waters XevoG2-XS QToF MS and UPLC in MS^e mode, phase I metabolites were investigated and ranked according to their relative abundance. UNIFI software (Waters Corporation) was applied to search for metabolites and to localize the metabolic transformations. The localization is based on the fragmentation pattern induced by high energy collision.
3. The development of the routine confirmatory analysis on a Shimadzu LCMS-8050 triple quadrupole mass spectrometer was based on the quantitation of the commercially available reference standard of (9R)-11-COOH-HHC and the investigation of its glucuronide conjugated form that was produced *in vitro* from the commercially available reference standard of (9R)-11-COOH-HHC. Urine samples from HHC users (n=31) were hydrolyzed in three ways and quantified. The presence of the glucuronide form and the concentration of the unconjugated form determined the efficiency of each hydrolysis method. Besides, the urinary concentration level to be monitored for (9R)-11-COOH-HHC was determined using the most efficient hydrolysis method. The extraction method was developed according to the sensitivity of the LC-MS/MS and the required concentration level.

II. Materials and Methods

a) Reagents and chemicals

The Department of Drug Investigation of Hungarian Institution of Forensic Sciences provided the reference standard containing the mixture of (9R)-HHC and (9S)-HHC. Based on the exact molecular mass, the exact mass of generated fragments, and observed retention time that had been set based on the reference standard (9R)-HHC was identified in the jelly gum purchased under the name 'HHC Premium Gummies' from Eighty8 (Czech Republic), that was used for self-administration. The extracted ion chromatograms belonging to the protonated HHC molecules are illustrated in *Figure 1*. (9R)-11-COOH-HHC reference standard (1 mg; purity:≥98%) was purchased from Cayman Chemicals. Incubation reagents [superoxide dismutase enzyme, UGT-system (Solution A and B), NADPH-regenerating system (Solution A and B), 0.5 M potassium phosphate buffer (pH 7.4)] were purchased from Corning (New York, USA). Abalonase ULTRA

hydrolysis enzyme kit, Escherichia coli β-D-glucuronidase IX-A hydrolysis enzyme and BiotageIsolute C18 SPE-cartridge (200 mg, 3 mL) was obtained from Shimkon Corporation (Budapest, Hungary). Leucine Enkephalin for Lockspray solution was obtained from Waters Corporation (Milford, USA) as a lyophilized peptide. Solvents [methanol, acetonitrile, ultrapure water, formic acid (LC-MS grade), acetic acid (100%)], KOH, KCl, and $MgCl_2$were purchased from Merck (Taufkirchen, Germany). Pooled human S9 fraction (pS9, 50 donors) and pooled human liver microsomes (pHLM, 50 donors) were purchased from Corning (New York, USA) and stored at -80°C untiluse.

b) In vitro incubation with pHLM and pS9

First, to tentatively identify a carboxylic acid metabolite in the self-administration urine to develop an appropriate screening method, and second, after identifying HHC-positive authentic urine samples, to investigate the metabolic profile of HHC, *in vitro* experiments with pHLM and with pS9 were performed. Both experiments were performed in five replicates where the fifth reaction mixtures lacked the substrate compound and were analyzed as blank *in vitro* samples. The reaction mixture consisted of either (*i*) pHLM (5 μL, 20 mg/mL), NADPH-regenerating system – Solution A (5 μL) and Solution B (1 μL), superoxide dismutase (10 μL, 3 kU/mL) and potassium phosphate buffer (20 μL, 0.5 M, pH 7.4) in 60 μL water, or (*ii*)pS9 (10 μL, 20 mg/mL), NADPH-regenerating system – Solution A (5 μL) and Solution B (1 μL),K/Mg solution (2 μL, 8mM $MgCl_2$ and 33 mM KCl) and potassium phosphate buffer (20 μL, 0.5 M, pH 7.4) in 60 μL water. 1 μL of the parent compound (10 mg/mL in methanol) was added and incubated for 120 min at 37°C. 300μL ice-cold acetonitrile was added to terminate the reaction. After centrifugation (14500 rpm, 4°C), the supernatant was analyzed with the LC-MS-QToFMS^e method.

c) Urine samples from a volunteer consumer and the development of a screening method

A 38-year-old female volunteer consumed one piece of the jelly gum called 'HHC Premium Gummies' from Eighty8, reported to contain '25 mg HHC/piece'. The methanol extract of the jelly gum was analyzed with LC-MS-QToF. The exact molecular mass, the exact mass of generated fragments and retention time had already been set based on the hexahydrocannabinol reference standard. (9R)-HHC was identified in the jelly gum.

First, 5-5 ml aliquots of collected urine samples from the volunteer consumer (0-8 h and 8-16 h) were hydrolyzed by Abalonase ULTRA hydrolysis enzyme and extracted by solid phase extraction (Isolute C18 SPE-cartridge, 200 mg, 3 mL) applying a 25-fold concentration. The concentrated extracts and the *in vitro* samples were analysed with LC-MS-QToF to identify a carboxylic acid metabolitetentatively and to recognise its retention time. In the next step, the chromatographic analysis applied on the LC-MS-QToF was transferred to the LC-MS/MS used for screening. The retention time and the mass of the parent ion of the carboxylic acid metabolite was known from the QToF identification. So automatic MRM-optimization supported by chromate-graphic separation could be applied to the LC-MS/MS. By the optimised MRM-transitions, the retention time for the carboxylic acid metabolite could be determined for the screening analysis on the LC-MS/MS as well.

Second, to 100-100μL aliquots of collected urine samples from the volunteer consumer (0-8 h and 8-16 h), 300 μL ice-cold acetonitrile was added, and after centrifugation (14500 rpm, 4°C), the supernatant was analyzed by the LC-MS/MS applying the chromatographic method used for screening and the optimized MRM-transitions for the carboxylic acid metabolite. However, the MRM-transition peak in these unhydrolyzed samples was supposed to belong to the glucuronic acid conjugate of the above analyzedcarboxylic acid metabolite. The retention time and the mass of the parent ion of the glucuronide-conjugated carboxylic acid metabolite was determined in the unhydrolyzed samples. Soits automatic MRM-optimization supported by chromatographic separation could also be applied.

The optimised MRM-transitions and appropriate retention time data for the glucuronic acid conjugate of a carboxylic acid metabolite was introduced in the routine qualitative screening method for illicit drugs and new psychoactive substances.

All steps of the automatic MRM-optimization process supported by chromatographic separation for the two metabolites on the LC-MS/MS system are illustrated in *Figure 2*.

d) Authentic urine samples for metabolic profiling and the development of a routine confirmatory method

Urine samples analysed in this study, have been submitted to our laboratory for forensic drug analysis. They were stored at 4°C until analysis.

The optimised MRM-transitions of the mentioned glucuronide-conjugated carboxy-metabolite and its retention time were imported into the routine screening analysis for new psychoactive substances and other common drugs of abuse. All urine samples submitted to our laboratory for forensic drug analysis were screened by this improved method using liquid chromatography with tandem mass spectrometry (LC-MS/MS) from April 2023.

Samples were only used for further experiments if the HHC-metabolite positive screening was followed by HHC-epimer positive confirmation. The presence of the HHCepimers was confirmed in highly concentrated samples using liquid-chromatography in conjunction with high-resolution mass spectrometry (LC-MS-QToF).

Eleven urine samples analysed in the metabolismstudy and thirty-one urine samples used in the development of a routine confirmatory method were screened positive for the optimised glucuronide-conjugated metabolite. All were confirmed positive for HHC epimers. One analyte-free urine sample was obtained from a person who had not taken any drugs or medication and who was screened and confirmed negative for the optimized glucuronide conjugate of the (9R)-HHC-carboxylic acid metabolite and HHC epimers, respectively.

e) Instrumentation and sample preparation

i. *The LC-MS/MS screening method*

In the Department of Forensic Toxicology at the Hungarian Institute of Forensic Sciences, a routine qualitative screening analysis is applied for new psychoactive substances and other common drugs of abuse. This LC-MS/MS method, monitoring 362 compounds, is described in detail in our previous work (10). This screening method was improved in this present work. MRM-transitions were optimised for the appropriate HHC metabolites and they were imported into the analysis method.

ii. *The LC-MS-QToF qualitative confirmatory method and metabolic profiling method*

In the confirmatory method, 1 ml aliquots of urine samples were hydrolyzed with Abalonase ULTRA hydrolysis enzyme according to the application guide. 1 mL of 1:9:2.5 (v/v/v) hydrolysis buffer/water/hydrolysis enzyme solution was added to 1 mL aliquots of urine samples and incubated for 30 min at 60 °C. Our routine extraction method developed for the extraction of cannabinoids from blood was used for the qualitative confirmation of HHC consumption. 2 mLacetonitrile and 3 mL of 0,01 M HCl-solution were added to 1 mL aliquots of urine or blood samples and vortexed. The solid phase extraction procedure on Biotagelsolute C18 SPE-cartridges was done by the Automated Biotage Rapid Trace SPE-Workstation System. Sample was loaded to column with 1 mL/min flow rate after column activation with 1 mL methanol and conditioning with 1 mL 0.01M HCl-solution. After washing the column with 1 mL water and drying under nitrogen gas for 10 minutes, the analyte was collected by elution with 2.4 mL 2:1 (v/v) hexane/ethyl-acetate with 1 mL/min flow rate. Five replicates of each sample were extracted. The five extracts were united at the end of the SPE procedure. The extraction solvent was vaporized, and the united extracts were reconstituted into 200 µL 1:1 (v/v) methanol/water.

For the metabolic profiling study, *in vitro* samples were precipitated with acetonitrile. Authentic urine samples were extracted by the above characterised HHC confirmatory extraction method.

Extracts were analyzed using a Waters ACQUITY I-Class UPLC system coupled with a Xevo G2-XS QToF system from Waters Corporation. Chromatographic parameters of the Forensic Toxicology Application developed by Waters Corporation for LC-MS-QToF were applied. The details of the chromatographic separation and the working conditions of the high-resolution mass spectrometer were presented in our previous work (10). UNIFI software performed the high-resolution data processing.

iii. *The LC-MS/MS quantitative confirmatory method*

The routine quantitative confirmation of HHC consumption is based on the quantitation of (9R)-11-COOH-HHC, since its reference standard is commercially available. However, this metabolite is supposed to be excreted in a conjugated form to a high extent, similarly to THC- and CBD-metabolites. Therefore, in order to have a correct quantitation of the unconjugated form, first, an efficient hydrolysis is essential.

Three hydrolysis methods were compared. Four aliquots of the thirty-one authentic urine samples proved positive for HHC were used. In case of three aliquots the following three hydrolysis methods were applied. One aliquote remained unhydrolyzed. Ice-cold acetonitrile containing two internal standards was added in 3:1 (*v/v*) ratio to the urine samples. The supernatant was analysed after centrifugation at 14500 rpm at 4°C.11-nor-Δ9-tetrahydrocannabinol-9-carboxylic acid-D3 and 11-nor-Δ9-tetrahydrocannabinol-9-carboxylic acid glucuronide-D3 were used as internal standards.

1. Abalonase ULTRA enzyme hydrolysis

100 µl aliquots of urine samples were hydrolyzed with Abalonase ULTRA hydrolysis enzyme according to the recommendations.100 µlof 1:9:2.5 (v/v/v) hydrolysis buffer/water/hydrolysis enzyme solution was added to 100 µl aliquots of urine samples and incubated for 30 min at 60 °C.

2. Escherichia coli β-D-glucuronidase IX-A enzyme hydrolysis

100 µl aliquots of urine samples were hydrolyzed with Escherichia coli β-D-glucuronidase IX-A hydrolysis enzyme according to the recommendations. 6 µl of hydrolysis enzyme was added to 100 µl aliquots of urine samples and incubated for 90 min at 37.5 °C.

3. Alkaline hydrolysis

100 µl aliquots of urine samples were hydrolyzed with alkaline hydrolysis. 5 µl of 10 M KOH was added to 100 µl aliquots of urine samples and incubated for 15 min at 60 °C. After the incubation, 7 µL of 100% acetic acid was added to each aliquot.

The comparison of the efficiency of the different hydrolysis methods had two aspects: (9R)-11-COOH-HHC was quantified and the presence of (9R)-11-COOH-HHC-glucuronide was monitored. The calibration points were 25 ng/mL, 50 ng/mL, 100 ng/mL, 250 ng/mL and 500 ng/mL.

The LC-MS/MS method for cannabinoids was used for the routine quantitative confirmation of HHC consumption. Ice-cold acetonitrile containing two internal standards was added in 1:3(*v/v*) to the hydrolysed samples. The supernatant was analysed after centrifugation (14500 rpm, 4°C). 11-nor-Δ9-tetrahydrocannabinol-9-carboxylic acid -D3 and 11-nor-Δ9-tetrahydrocannabinol-9-carboxylic acid glucuronide-D3 were used as internal standards.

Extracts were analysed using a Nexera UPLC coupled with an LCMS-8050 Triple Quad mass spectrometer from Shimadzu. Chromatographic separation was identical to the above mentioned screening method.

The triple quadrupol mass spectrometer was equipped with an electro spray ionisation source. The mass spectrometer can work in positive (ESI+) or in negative (ESI-) ionisation mode. The ionisation mode depends on the optimised MRM-transition. The interface temperature was 250 °C, the heat block temperature was 300 °C, the DL temperature was250 °C and the desolvation temperature was 444°C. Nitrogen was used as drying gas and nebulizing gas. Argon was used as collision gas.

Acquiring(9R)-11-COOH-HHC and its glucuronide conjugated form was performed in positive MRM mode. The precursor ion of (9R)-11-COOH-HHC is m/z 347.40, the quantifier product ion is m/z 193.25, the qualifier product ions are m/z 121.35 (with reference ion ratio 66.39% ± 30%) and m/z 207.30 (with reference ion ratio 50.41% ± 30%) with event time of 0.016 sec. The set time window is 5.900 min – 6.500 min. According to the optimized fragmentation, in case of (9R)-11-COOH-HHC-glucuronide, the precursor ion is m/z 523.40, the quantifier product ion is m/z 347.40, the qualifier product ions are m/z 329.30 (with reference ion ratio 18.69% ± 30%) and m/z 207.30 (with reference ion ratio 26.36% ± 30%) with event time of 0.016 sec. The set time window is 5.200 min – 5.800 min. Data processing was performed using Lab Solutions software.

III. Results and Discussion

a) The improvement of a routine screening analysis

Figure 2. shows the steps of the optimisation process for the fragmentation of the carboxy-metabolite. This metabolite and its glucuronide-conjugated form were identified in the self-administration urine by high resulotion mass spectrometry analysis. The MRM-transitions of the glucuronide-conjugated carboxylic acid metabolite were imported into the routine urinary screening analysis. Acquiring the fragment ions of the glucuronide-conjugated carboxylic acid metabolite was performed in positive MRM mode. According to its optimised fragmentation, the precursor ion is m/z 523.20, the quantifier product ion is m/z 347.10, the qualifier product ions are m/z 329.30 (with reference ion ratio 25.23% ± 30%) and m/z 207.00 (with reference ion ratio 49.85% ± 30%) with event time of 0.016 sec. The set time window is 5.100 min – 5.500 min. Data processing was performed using Lab Solutions software.

From April 2023 to June 2023, 2118 urine samples were screened in our laboratory for forensic drug consumption, and nine authentic urine samples were screened positive for the glucuronide conjugated carboxylic acid metabolite. All were qualitatively confirmed by identifying the parent compounds by the QToF analysis. The nine authentic samples and the two self-administration samples were investigated in the metabolic profiling assay.

From April 2023 to September 2023, 5012 urine samples were submitted to our laboratory for forensic drug analysis. Twenty-nine authentic urine samples were screened positive for the glucuronide-conjugated carboxylic acid metabolite. All were qualitatively confirmed by identifying the parent compounds by the QToF analysis. These twenty-nine samples and the two self-administration samples were investigated in the development of a quantitative confirmatory analysis.

b) The metabolic profiling assay

i. *Analytical strategy for metabolic profiling*

UPLC-HRMS MS^e data was processed by UNIFI software in the following way.

First, the 'Transformations' tool of UNIFI software offers a list of possible metabolic transformations. Based on literature data, the metabolic profile of similar compounds, and the structure of HHC, the following biotransformations were selected: reduction (+H2, Phase I), oxidation (+O, Phase I), desaturation (-H2, Phase I), deethylation (-C2H4, Phase I), carboxylation (-H2+O2, Phase I). The software generated the chemical formula of all the potential metabolites that might be formed from the parent compound by these transformations and their combination.

Second, the protonated molecule of these possible metabolites might be detected in the low collision energy channel of the MS^e acquisition. Therefore, extracted ion chromatograms for these ions with a mass window of 20 ppm were automatically generated. All chromatographic peaks in the low collision energy data and the high collision energy spectra belonging to these peaks were investigated by the software.

In the third step, all chromatographic peaks were filtered for mass error being between -5 ppm and +5 ppm and the results of the positive control samples were compared to the blank and the *in vitro* samples. In case, the chromatographic peak was not detected in the blank samples and mass accuracy is appropriate, the peak was accepted as a possible metabolite and the high collision energy spectrum had to be analysed.

The high collision energy channel of the MS^e acquisition is practically the fragmentation pattern of the molecule. These spectra were also automatically processed. It means that the chemical formula of the fragments was calculated from their accurate mass and the software suggested a structure for the fragments based on their chemical formula and the parent compound. Only one transformation could be automatically processed and localized by the software. Therefore, *in silico* suggestion for the structural formula of the entire molecule was provided only for metabolites generated from one biotransformation. Therefore, the detailed analysis of the estimated chemical formula of the fragments was the following step. In case the fragmentation pattern was obvious, the position of the metabolic transformation could be determined, and the structure of the potential metabolite was suggested.

Following these steps, the extracted ion chromatograms belonging to the protonated molecule of the potential metabolites based on the expected biotransformations and their combination with a mass window of 20 ppm, the high collision energy MS/MS spectra belonging to the integrated peak on the extracted ion chromatograms, the estimated structure of the fragments identified in the fragmentation pattern and the estimated structure of the possible metabolite based on the estimated structure of the identified fragments are illustrated on *Figure 3., 4., 5. and Table 1., 2., 3., 4., 5.*

Based on retention times and molecular structure, ionization efficiency can change due to matrix effects (11, 12). However, concerning each ionization technology, a general prevalence and relative abundance of metabolites can be provided by comparing MS peak areas. *Figure 6. and 7.* summarize the relative amount of the 13 metabolites and the HHC epimers detected in the eleven urine samples from HHCusers, in the *in vitro*p HLM and in the *in vitro* pS9 samples. The relative abundances were calculated by averaging the ratio of the areas of each compound related to the most prevalent compound in each sample.

ii. *Fragmentation pathway of (9R)-HHC and (9S)-HHC*

Figure 8. shows the structure and fragmentation pattern of the two HHC epimers, (9R)-HHC and (9S)-HHC, based on the conformation of the C-11 methyl group. *Table 6.*shows the elemental composition, the estimated structure and the observed m/z with the mass error in ppm of the 12 most intensive high energy fragments generated from the two HHC epimers. The most intensive product ions are identical and all correspond to the stable alicyclic system of the parent compound. However, the difference in the retention time of the two epimers assures the specific identification of the two compounds.

iii. *Metabolite identification*

Metabolite identification was based on

- the extracted ion chromatograms of the protonated molecule of the potential metabolites based on the expected biotransformations and their combination with a mass window of 20 ppm (*Figures 3., 4., 5.)*
- the high collision energy MS/MS spectra belonging to each integrated peak on the extracted ion chromatograms (*Figures 3., 4., 5.)*
- the estimated structure of the fragments identified in the fragmentation pattern (*Tables 1., 2., 3., 4., 5.*)

The estimated structure of a metabolite was presented in *Figures 3. and 5.,* if the identified fragments belonging to that metabolite were unambiguously defined.

iv. *The oxidised [HHC+O] metabolites(Figure 3. and Tables 1., 2., 3.)*

M01: The molecular formula of M01 (m/z 333.2422; C21H32O3) corresponded to oxidation. The most intense product ion at m/z 203.0705corresponding to C12H11O3, and the product ion at m/z 197.1190 corresponding to C11H13O3 indicated that all the three oxygen atoms are connected to or part of the chroman ring. It means the oxidation might be on the C-2 or the C-4 methyl-group.

M02 – M05: The coeluting peaks of M02, M03, M04 and M05 were observed at m/z 333.2422 corresponding toC21H32O3suggesting an oxidation. The observed fragmentation indicated that the hydroxyl group was on the alkyl side chain, since the most intensive product ions at m/z 259.1693 (C17H23O2)and at m/z 231.1377 (C15H19O2) reveal that the hexahydrobenzochromene three-ring system has only two oxygen atoms. It means the oxidation might be on the C-1', C-2', C-3', C-4' or the C-5' carbon of the n-pentyl side chain.

M06: M06 was detected at m/z 333.2422 (C21H32O3) corresponding to an oxidation. Product ion at m/z 193.1227 (C12H17O2)excludes the oxidation of the chroman ring and the n-penthyl side chain. Product ion at m/z 259.1695 (C17H23O2) excludes the oxidation of the three-ring system. Therefore, the oxidation might be on the C-11 methyl-group.

v. *The carboxylated [HHC-H2+O2] metabolites (Figure 4. and Table 4.)*

The fragmentation patterns of the carboxylated metabolites are very similar. The product ions are identical. Only their ratio might be specific for the metabolite. Therefore, the exact structure of most carboxylated metabolites and the location of the metabolic transformation could not be determined. *Table 4.* lists the elemental composition, the possible structure and the observed exact mass of the most intensive, unspecific high energy fragments generated from the carboxy-metabolites. *Figure 4.* shows the high

energy spectrum and the chromatogram of the metabolites. Only two of them could be tentatively (M07) or unambiguously (M11) identified.

M07: The molecular formula obtained for M07 (m/z 347.2219; C21H30O4) corresponds to a carboxylated metabolite. Product ion at m/z 188.0471 (C11H8O3) suggests the carboxylation was on one of the geminal methyl groups on the hexahydrobenzochromene core.

M11: M11 was proved to be (9R)-11-COOH-HHC by investigating the commercially available reference standard.

vi. *The double carboxylated [HHC+2(-H2+O2)] metabolite (Figure 5., Table 5.)*

M13: M13 corresponds to a double carboxylated metabolite based on its elemental composition (m/z 377.1997; C21H28O6). Product ions m/z 197.0820 (C10H13O4), m/z 184.0743 (C9H12O4)and m/z 237.1125 (C13H17O4) contain four oxygen atoms, relatively few carbon atoms and a relatively higher number of hydrogen atoms. This strictly determines the possible conformation of the product ions based on the structure of the parent compound. The only possible conformation of the metabolite according to these product ions with four oxygens is the double carboxylated, C-11-carboxy- and C-12- or C-13-carboxy-HHC.

Reference standards are essential for further confirmation to compare the retention time and fragmentation pattern of our results and the standards. Furthermore, non-official standard compounds have to be analyzed by additional analytical techniques such as nuclear magnetic resonance(13).

vii. *Metabolite ranking*

Figure 6.and 7. summarize the relative amount of the 13 tentatively identified metabolites and the HHC epimers detected in the eleven urine specimens from HHC users, detected in the pHLM samples and in the pS9 samples, respectively.

In case of the development of the screening method, the *in vitro* results served as a base for importing only the M10 carboxylated metabolite and its conjugated form into the analysis method, since only this carboxylated metabolite was present in the pHLM and pS9 samples. However, the results of the *in vitro* metabolite ranking study are always less reliable, since the metabolic transformations and pathways are entirely determined by the composition of the incubation mixture. These systems usually lack extrahepatic pathways. The results of the *in vitro* metabolite ranking might be informative and they might be compared to the *in vivo* ranking, however, metabolite profiling is usually based on the *in vivo* results.

According to the metabolite ranking study of the *in vivo* samples, all metabolites were more abundant than the parent compound having a mean ratio of 31.98%. In six urine samples the most abundant metabolite was M11 (mean ratio: 94.28%), in four sample M10 had the highest abundance (mean ratio: 65.66%) and in one sample M13 was the most abundant metabolite (mean ratio: 44.30%). M03, M04, M05, M06, M08, M09 and M12 were also dominant metabolites with a mean ratio of 23.29%, 33.75%, 25.83%, 21.35%, 24.68%, 31.97%, and 30.23%, respectively. The remaining three minor metabolites (M01, M02 and M07) have similar relative abundance to the parent compound.

In all the *in vitro* samples, the most prevalent compound was the parent compound. Four oxidised metabolites were detected in the *in vitro* samples: M02, M03, M05 and M06. Only one carboxylated metabolite, M10 was present in the pHLM and pS9 samples, in 9.46% and 1.95%, respectively. At the beginning of our study, this fact was the primary reason for importing M10 into the screening method. M01, M04, M07, M08, M09, M11 and M13 were present only in trace amounts. It is explained by the lack of specific support mechanisms in our *in vitro* systems (eg. the lack of the NADP-regenerating systems, that provides the essential cofactor in the THC-metabolism pathway needed in the production of the carboxy-THC metabolite (14)) or the *in vivo* role of extrahepatic pathways.

viii. *Metabolic pathways*

The estimated metabolic pathways of HHC-epimers in humans include oxidation of the chroman ring (M01), oxidation of the n-pentyl side chain (M02, M03, M04 and M05), oxidation of the C-11 methyl group(M06), carboxylation of one of the geminal methyl groups on the hexahydrobenzo chromenecore, carboxylation of the n-pentyl side chain, carboxylation of the C-11 methyl group (M07, M08, M09, M10, M11, M12), and the combination of two carboxylation steps (M13).

c) *The development of a quantitative confirmatory method – comparison of hydrolysis efficiency*

The routine quantitative confirmation of HHC consumption is based on the quantitation of(9R)-11-COOH-HHC. The glucuronide-conjugated metabolite was present in all non-hydrolysed samples. It proves that (9R)-11-COOH-HHC is partially excreted into urine in conjugated form, resulting a similar excretion profile to other cannabinoids. As a result, a hydrolysis step is essential for the quantification of the unconjugated form. One indicative aspect is the number of samples in which hydrolysis was complete (n=31).Complete hydrolysis meant that the glucuronide-conjugated form was undetectable in the samples. This number was 19, 1 and 3 for the Abalonase ULTRA enzyme, the *Escherichia coli* enzyme and the alkaline hydrolysis, respectively. The other indicative feature might be the concentration of the hydrolyzed samples. *Figure 9.* shows the average,

the minimum and the maximum concentration levels measured in the authentic samples. Abalonase ULTRA proved to be slightly more efficient in this aspect, than the two other methods. With Abalonase ULTRA enzyme hydrolysis, the average concentration of (9R)-11-COOH-HHC was 140 ng/mL, the highest concentration was 453 ng/mL and the lowest concentration was 0 ng/mL.

d) Comparison of the present work with metabolism studies of hexahydrocannabinol

The semi-synthetic cannabinoids, especially hexahydrocannabinol, got into the focus of numerous forensic studies in a short time after its appearance in 2022. The published metabolism studies are controversary. However, the controversial aspects of these studies might be understood if some fundamental aspects got clarified.

First, the results of *in vitro* studies are restricted by the composition of the *in vitro* system, especially the cofactors. Cofactor dependence experiments with human liver subcellular fractions revealed that 11-carboxy-THC and 7-carboxy-CBD formation largely depend on cytosolic NAD+-dependent enzymes, with lesser contributions from NADPH-dependent microsomal enzymes. Based on experiments with chemical inhibitors, aldehyde dehydrogenases contribute dominantly to 7-carboxy-CBD formation, and 11-carboxy-THC formation is partly mediated by aldehyde oxidase (14). It means that human liver microsome assays cannot produce the carboxy metabolites of CBD and THC. There is a high probability of the same situation for the carboxy metabolite(s) of HHC. As a result, the metabolism studies must not be based on the *in vitro* HLM assays, and the lack or low level of carboxy metabolites in the *in vitro* systems does not estimate the levels of carboxy metabolites in the *in vivo* samples.

Second, in case of *in vivo* samples, conjugation might mislead the study. Since glucuronide conjugated metabolites are not available commercially, their detection is complicated. However, urinary metabolite ranking can only be correct if the glucuronide conjugated metabolites are all monitored as well (phase 1 and phase 2 ranking), or if the investigation starts with a hydrolysis step (only phase 1 ranking).

The analytical equipment is also critical in the comparison of metabolite ranking studies. These a works aim to define a urinary concentration ranking. However, the sensitivity, meaning the ratio of the urinary concentration and the peak area in the analysis, might be proved to be constant in a linear range for each compound. Still, it is different for every compound in one analytical method, and it is different for one compound on different analytical equipment, as well. As a result, it might be utterly controversy if the ranking is based on a GC-MS analysis with EI ionization, where the parent compound is fragmented, and the base for the ranking is the sign of the main fragment, and the ranking based on an LC-HRMS analysis with ESI ionization, where the ionized parent compound is monitored, is compared.

Considering these aspects of metabolism studies regarding HHC, our work is compatible with numerous scientific works.

- The metabolites generated by the hydroxylation of the n-pentyl side chain were identified and found to be abundant in metabolism studies by GC-MS and LC-QToF (15, 16, 17).
- Deglucuronidation of urine samples was found to be essential in phase 1 metabolism studies in numerous works (15, 16, 17).
- Studies agree that the carboxy metabolites are present in hydrolisedurinary samples. However, some works detected them only in trace amounts (15, 16, 17) while other studies had similar results to ours and these metabolites were found to be abundant (19).
- (18), The use of chiral analytical column for the quantitative analysis of HHC in biological samples was proved to be advantageous in separating the epimers and their 11-carboxy and 11-hydroxy metabolites.
- Quantitative results were collected for urine, blood and oral fluid samples (19). Urinary concentration for (9R)-11-COOH-HHC is lower (c_{max}: 18.5 ng/mL) than in our study (c_{max}: 453 ng/mL) but the first result was obtained in non-hydrolysed samples.

IV. Conclusion

In this present paper we described the *in vivo* phase I metabolism of hexahydrocannabinol based on the LC-HRMS analysis of 11 authentic urine samples. The major biotransformations are oxidation (M1, M2, M3, M4, M5 and M6), hydroxylation (M7, M8, M9, M10, M11 and M12) and double carboxylation (M13). It should be noted that for metabolites other than M11, the chemical structures have not been unequivocally verified. Structure elucidation by interpretation of MS spectra provides strong clues to the chemical structure of the molecule. However, the structure should be verified by analysis of synthesized reference compounds or nuclear magnetic resonance analysis.

Another main result of this study is the ranking of the 13 *in vivo* HHC-metabolites. All were more prevalent than the parent compound, having a mean ratio of 31.98%. In six urine samples the most abundant metabolite was M11.In four samples M10 had the highest rank. In one sample M13 was the most abundant metabolite. M03, M04, M05, M06, M08, M09 and M12 were also dominant. The remaining three minor metabolites (M01, M02 and M07) have similar relative abundance to the parent compound.

We improvedour routine LC-MS/MS screening analysis by importing the optimised MRM-transition for

M10 and for its glucuronic acid conjugated form. These compounds had been identified by LC-HRMS analysis of biological samples and *in vitro* samples.

We developed an LC-MS/MS confirmatory analysis based on M11, a synthesized reference compound. We compared different hydrolysis methods by the quantitative analysis of M11 in 31 authentic urine samples. Hydrolysis with Abalonase ULTRA glucuronidase enzyme proved to be the most efficient method.

Conflicts of interest

The authors declare that there are no conflicts of interest

Data Availability Statement

All data are incorporated into the article.

Acknowledgement

The authors gratefully acknowledge scientists at the Department of Drug Investigation of Hungarian Institution of Forensic Sciences for their contributions.

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controlled administration of 25 mg inhaled HHC to seven volunteers. 60th International Association of Forensic Toxicologists Meeting, TIAFT 2023 O44

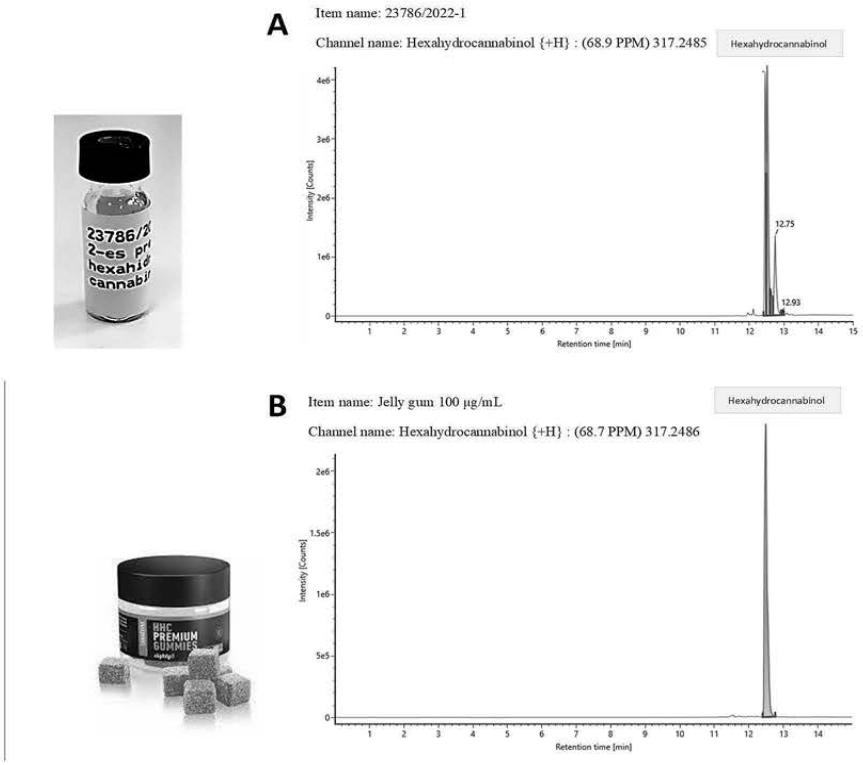
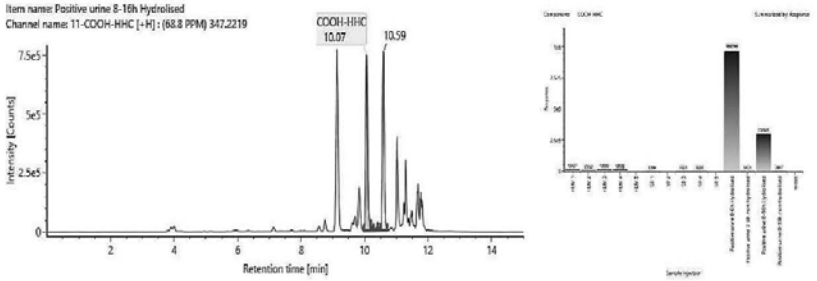
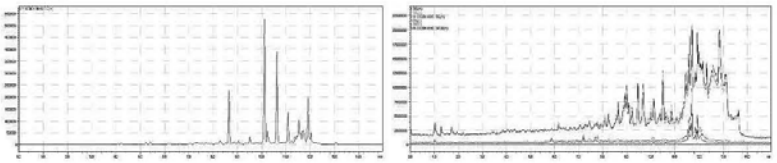


Fig. 1: The chromatogram of HHC epimers in the reference standard provided by the Department of Drug Investigation of Hungarian Institution of Forensic Science [A], and the chromatogram of (9R)-HHC in the jelly gum extract[B].

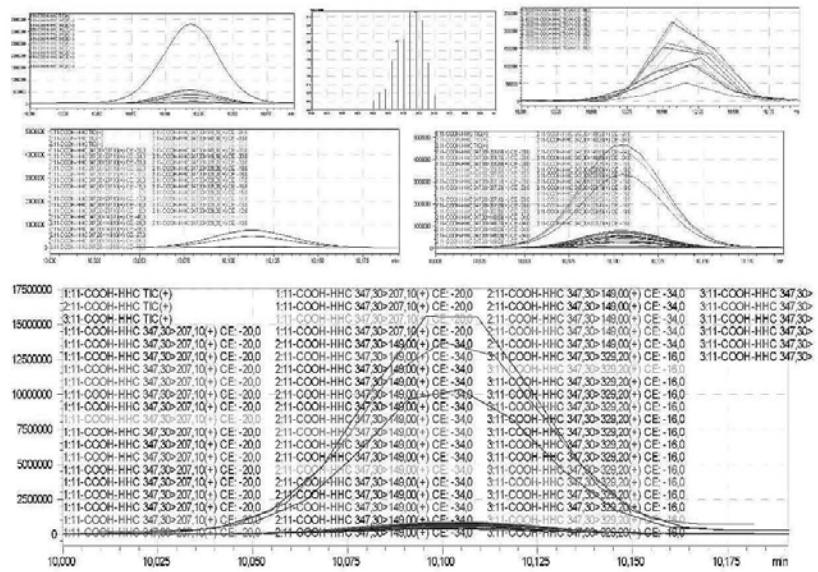
1. QToF analysis of the self-administration urine (hydrolysed/unhydrolysed, solid phase extraction, 25x concentration) detection of a carboxy metabolite in the urine sample and in the *in vitro* samples
   Rt: 10,07 min



2. LC-MS/MS analysis of the self-administration urine (hydrolysed, solid phase extraction, 25x concentration) after the method transfer; SIM and scan analysis
   Rt: 10,07 min

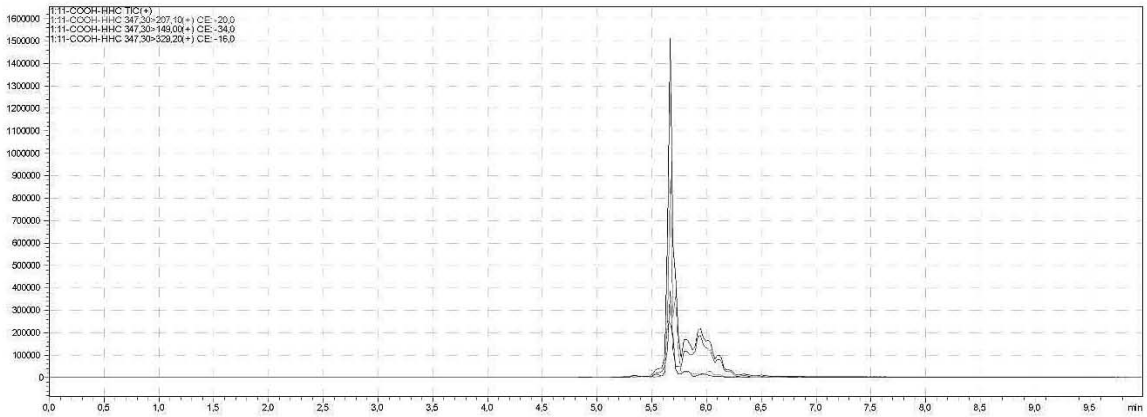


3. Automatic MRM-optimization supported by chromatographic separation on LC-MS/MS of the carboxy-metabolite identified by the QToF analysis in the self-administration urine (hydrolysed, solid phase extraction, 25x concentration)
   Rt: 10,07 min

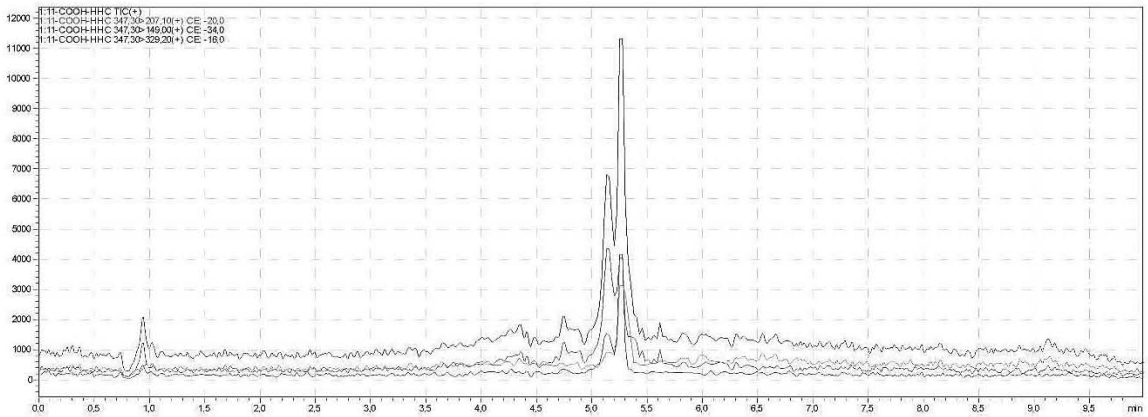


4. After importing the newly optimised MRM-transitions of the carboxy metabolite into the routine screening method, the self-administration urine was analysed following two different extraction procedures (hydrolysed, solid phase extraction, 25x concentration for carboxy-HHC; unhydrolysed, protein precipitation, 4x dilution for glucuronide conjugated carboxy-HHC)

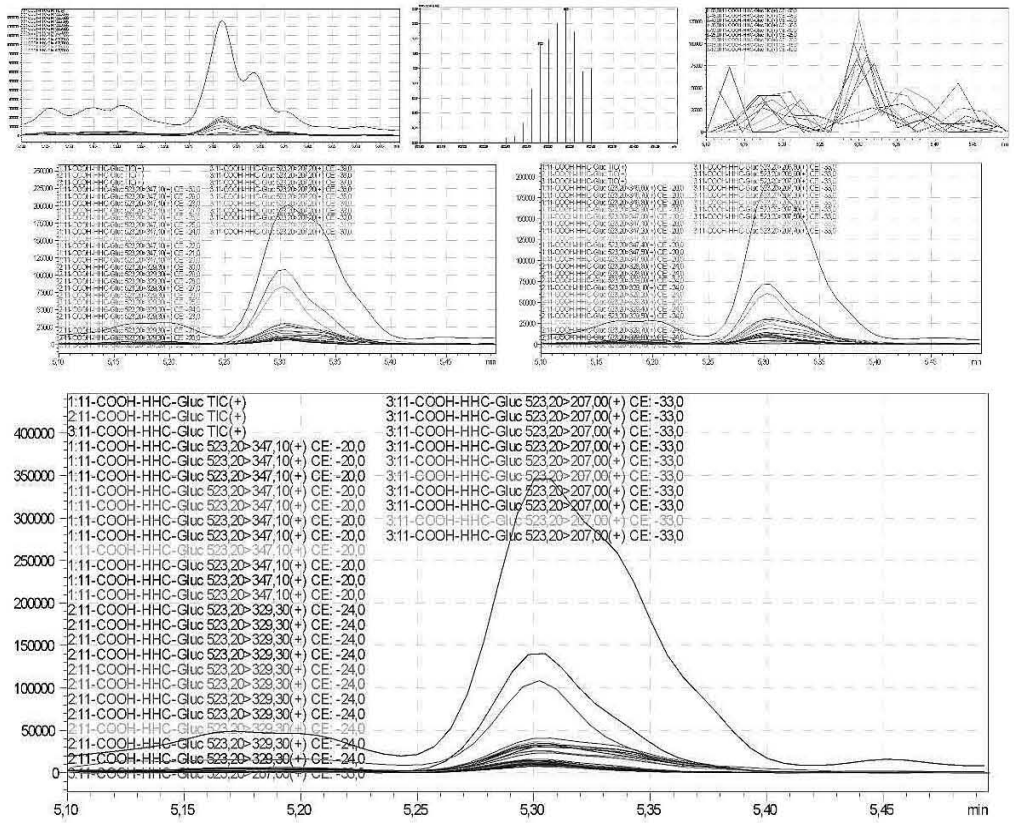
I. Hydrolysed, solid phase extraction, 25x concentration for carboxy-HHC
Rt: 5,667 min



II. Unhydrolysed, protein precipitation, 4x dilution for glucuronide conjugated carboxy-HHC
Rt: 5,300 min



5. Automatic MRM-optimization supported by chromatographic separation on LC-MS/MS of the carboxy-glucuronide-metabolite identified in the self-administration urine (unhydrolysed, protein precipitation, 4x dilution)
Rt: 5,30 min



6. As a result of the development of the screening method, the optimised glucuronic acid conjugated carboxy metabolite can be detected by the routine LC-MS/MS screening analysis

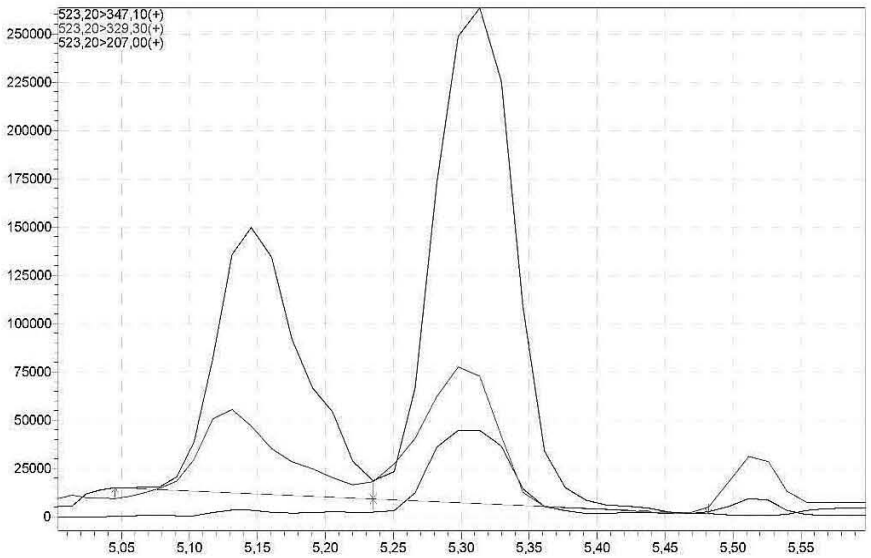
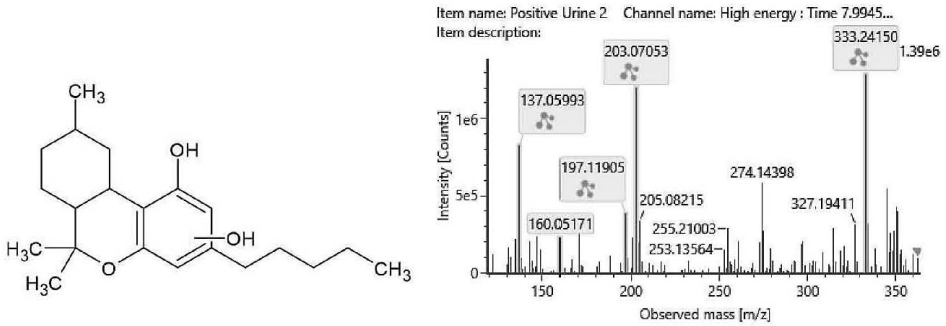
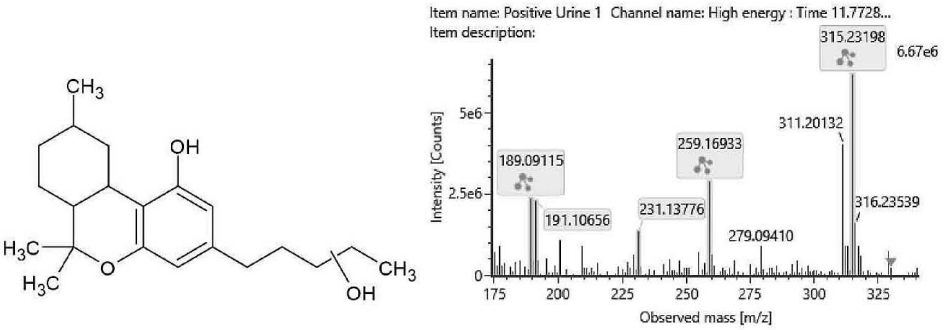


Fig. 2: The detailed development of the screening method

M01 (Rt: 7,98 min)



M02; M03; M04; M05 (in coelution)



M06 (Rt: 12,12 min)

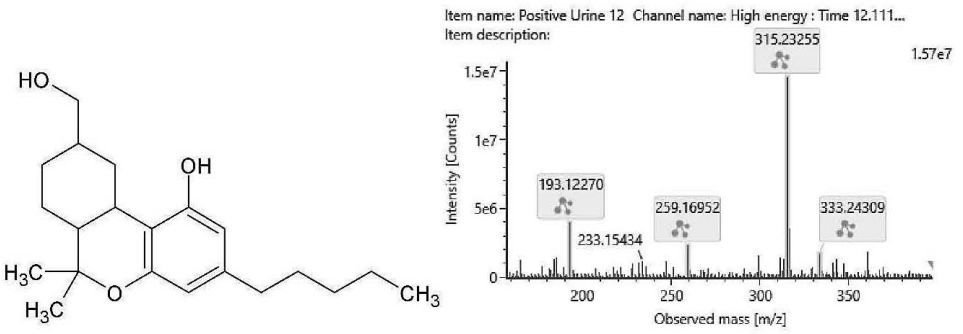
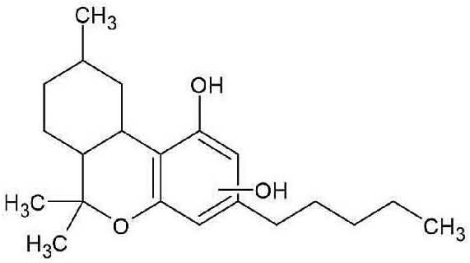
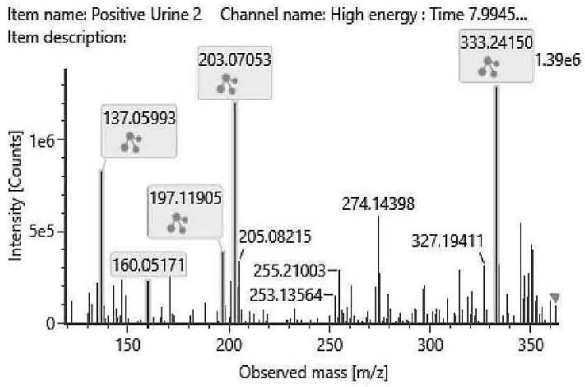


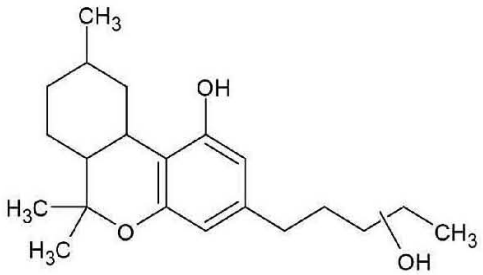
Fig. 3: The estimated structure, fragmentation pattern and chromatogram of the [HHC+O] metabolites (M01, M02, M03, M04, M05 and M06)

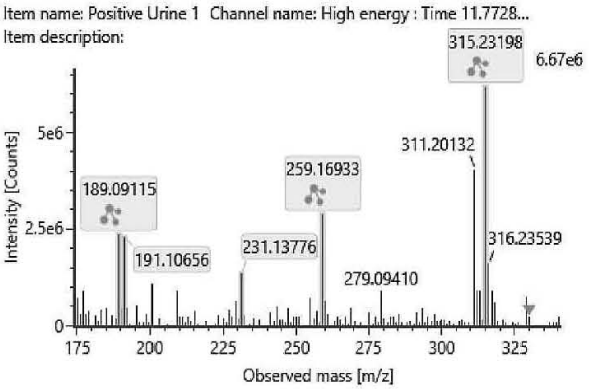
M01 (Rt: 7,98 min)



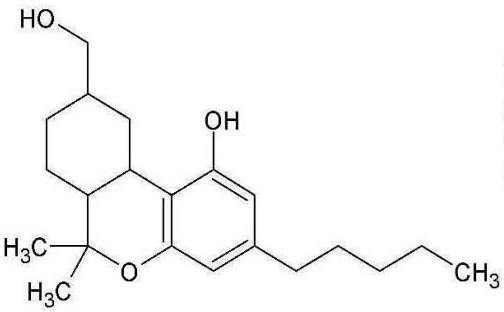


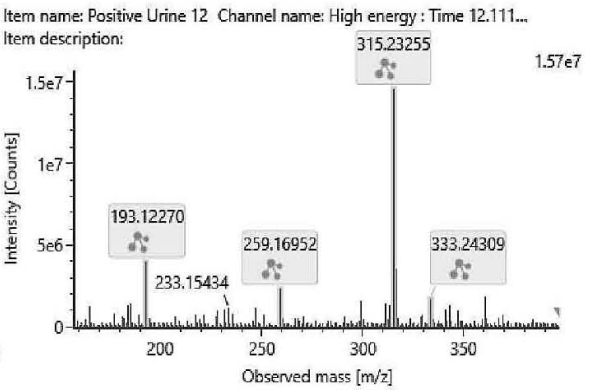
M02; M03; M04; M05 (in coelution)

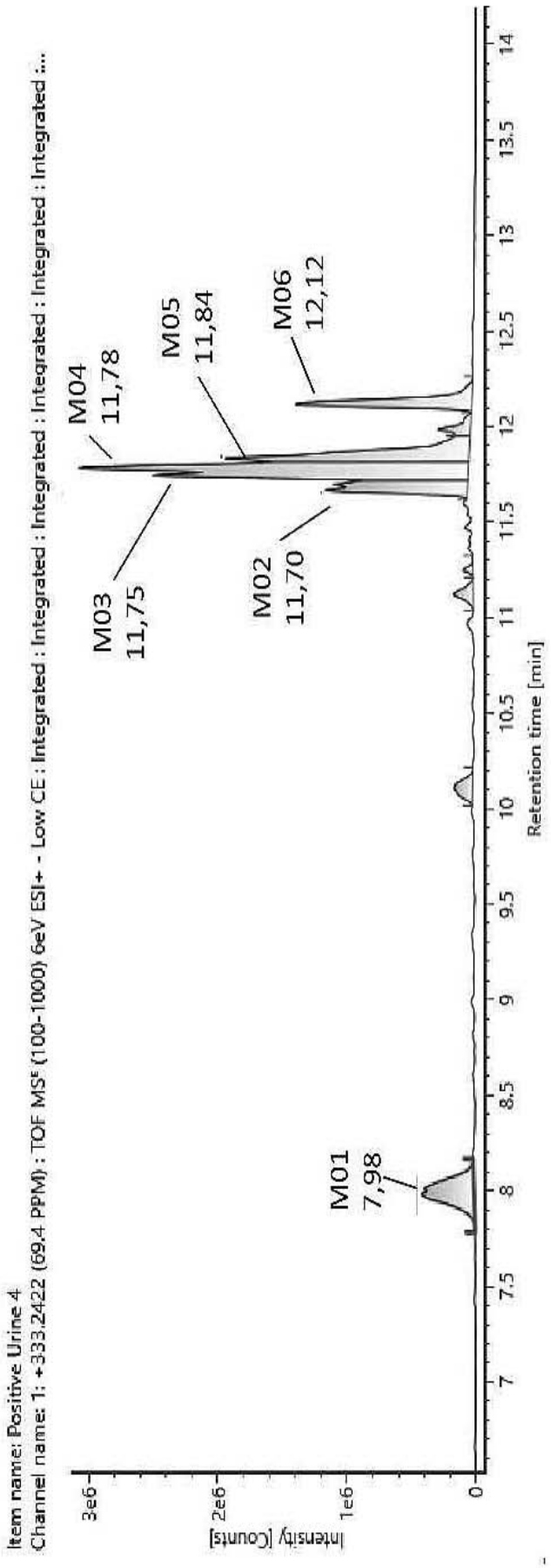




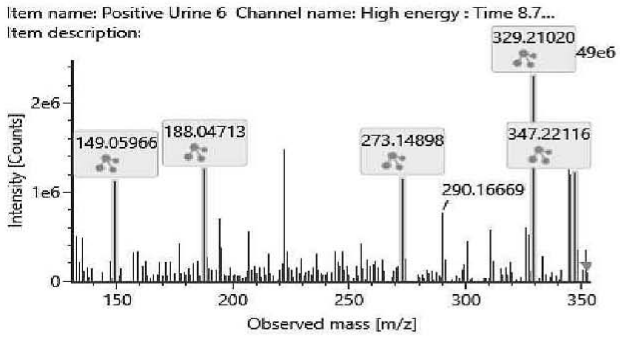
M06 (Rt: 12,12 min)



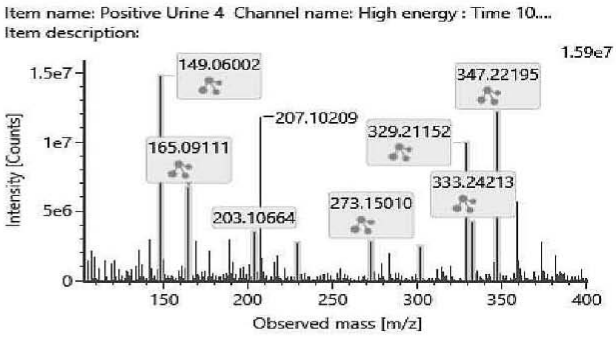




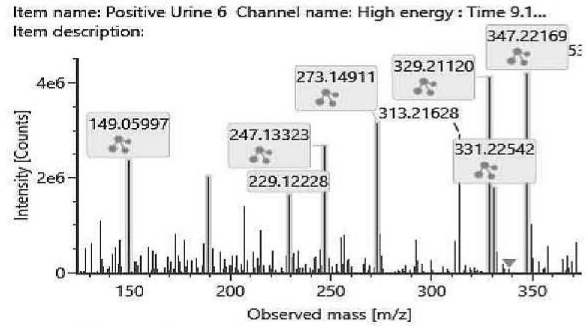
M07 (Rt: 8,75 min)



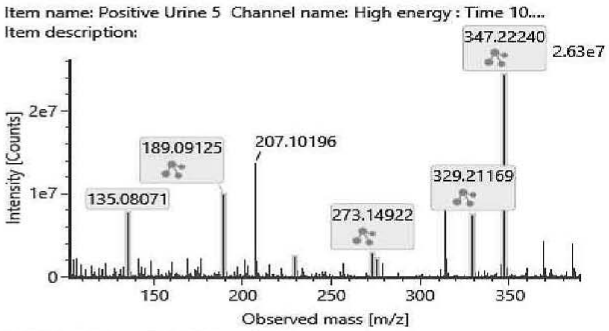
M10 (Rt: 10,07 min)



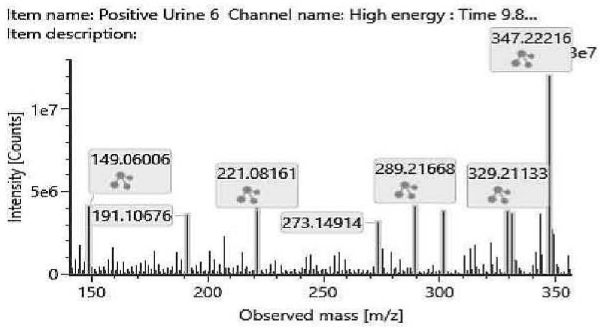
M08 (Rt: 9,14 min)



M11 (Rt: 10,58 min)



M09 (Rt: 9,84 min)



M12 (Rt: 11,03 min)

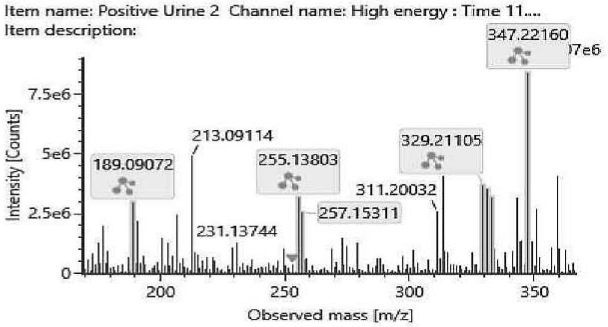
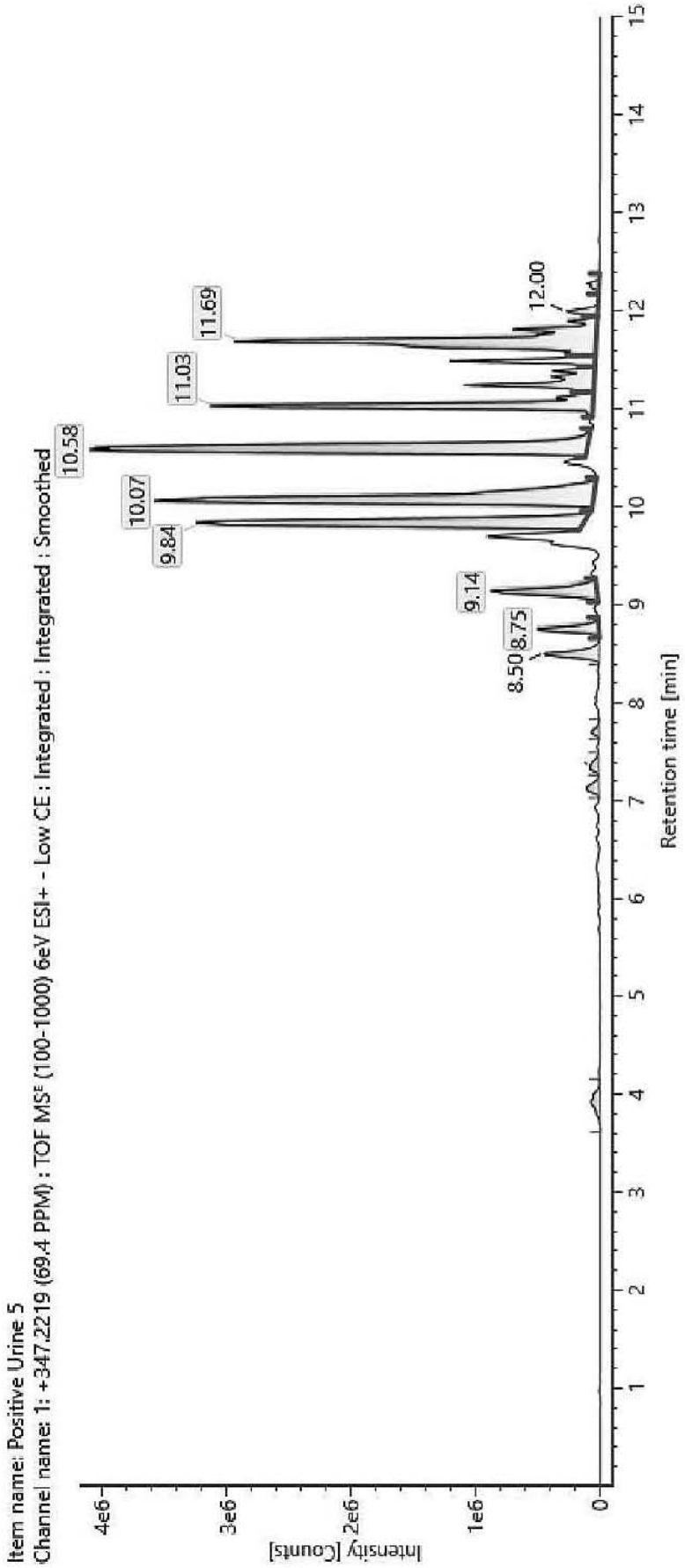


Fig. 4: The fragmentation pattern and chromatogram of the [HHC-H2+O2] metebolites (M07, M08, M09, M10, M11 and M12)



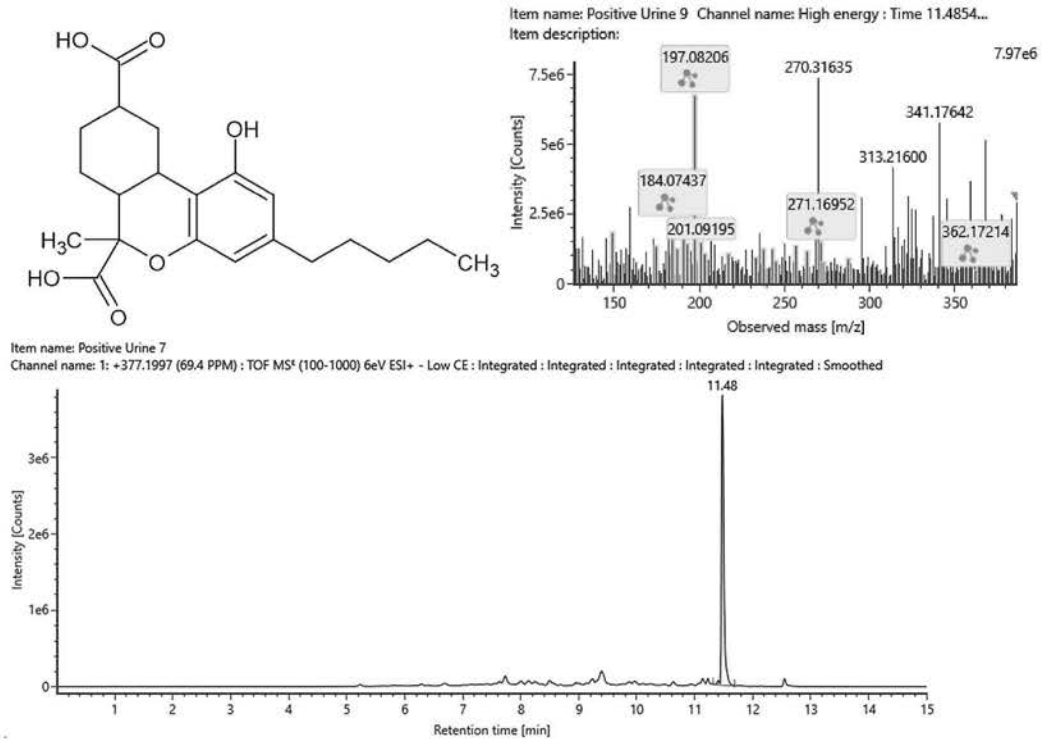


Fig. 5: The estimated structure, fragmentation pattern and chromatogram of M13 [HHC+2(-H2+O2) metabolite, Rt: 11,48 min]

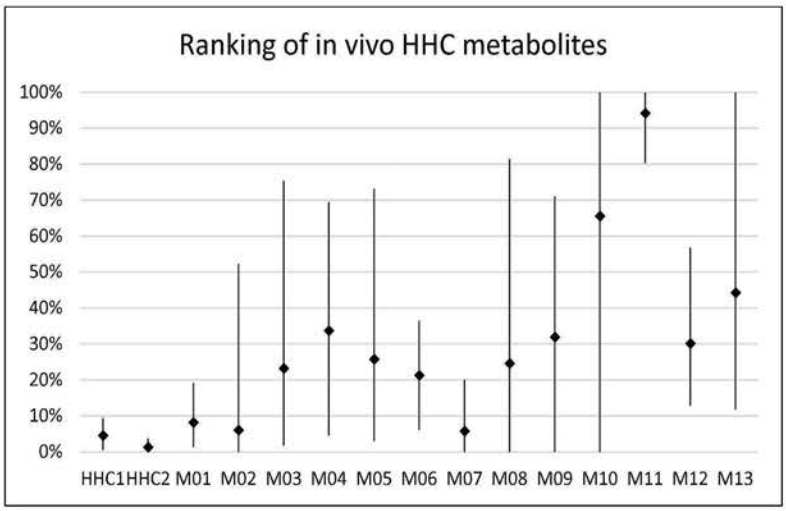


Fig. 6: The relative abundance of the tentatively identified metebolites and the parent compound(s) in the 11 urine specimens from HHC-users analysed

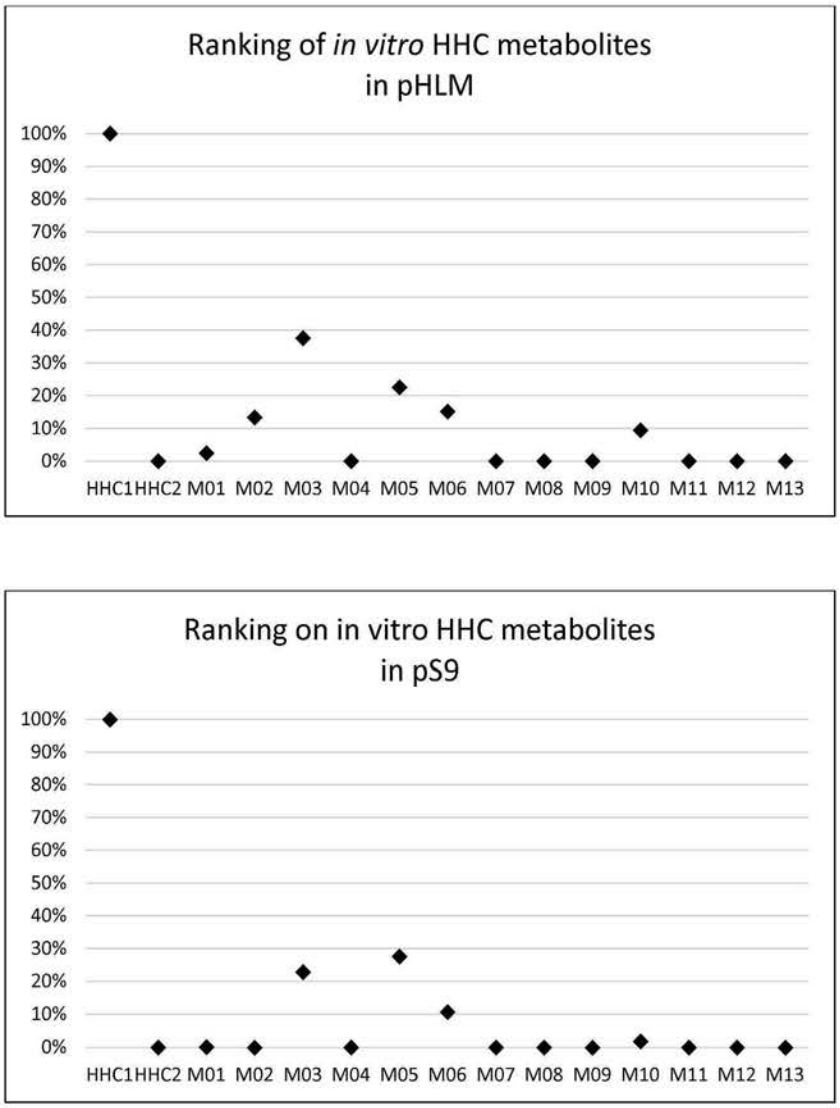


Fig. 7: The relative abundance of the tentatively identified metebolites and the parent compound in the pHLM and the pS9 samples

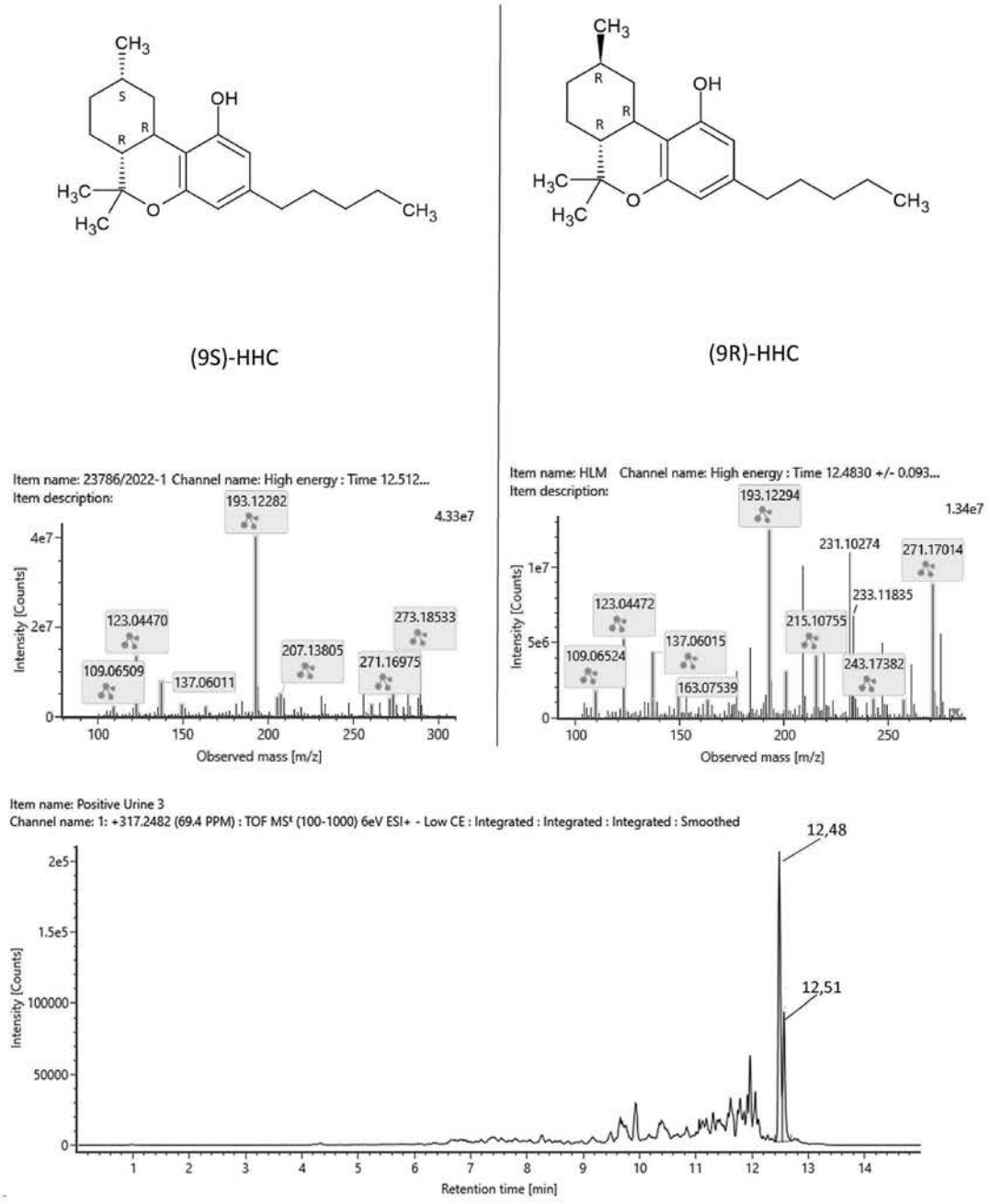


Fig. 8: The structure and fragmentation pattern of the two HHC epimers, (9S)-HHC and (9R)-HHC, based on the conformation of the C-11 methyl group

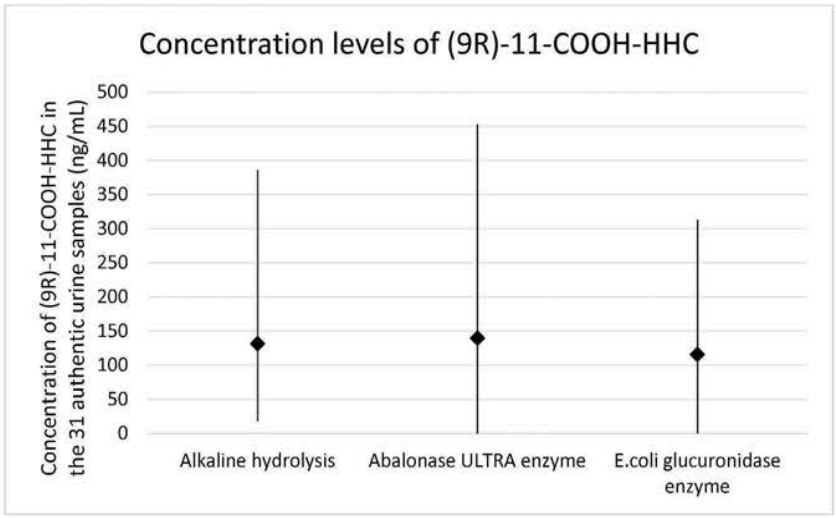


Fig. 9: Concentration levels in the thirty-one authentic urine samples quantified after the three different hydrolysis methods

Table 1: The elemental composition, the estimated structure, the observed m/z with the mass error in ppm compared to the expected m/z of the most intensive high energy fragments generated from M01

M01; [HHC+O] metabolite, Rt: 7,98 min

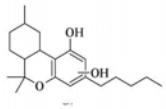
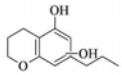
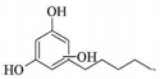
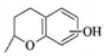
Observed m/z	Mass error (ppm)	Formula	Estimated structure
333.24150	-2.77	C21H33O3	
203.07053	1.29	C12H11O3	
137.05993	1.62	C8H9O2	
197.11905	9.29	C11H17O3	
160.05171	-1.07	C10H8O2	

Table 2: The elemental composition, the estimated structure, the observed m/z with the mass error in ppm compared to the expected m/z of the most intensive high energy fragments generated from M04, the metabolite with the highest peak among the four coeluting oxidised metebolites

M04; [HHC+O] metabolite, Rt: 11,78 min

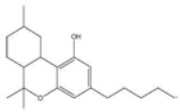
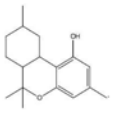
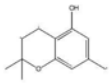
Observed m/z	Mass error (ppm)	Formula	Estimated structure
315.23199	0.41	C21H31O2	
259.16933	0.28	C17H23O2	
189.09115	0.77	C12H13O2	
191.10656	-0.48	C12H15O2	
231.13776	-0.87	C15H19O2	

Table 3: The elemental composition, the estimated structure, the observed m/z with the mass error in ppm compared to the expected m/z of the most intensive high energy fragments generated from M06

M06; [HHC+O] metabolite, Rt: 12,12 min

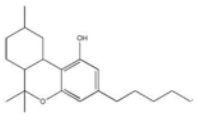
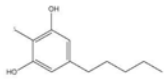
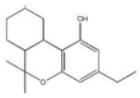
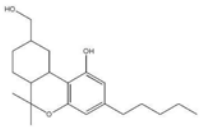
Observed m/z	Mass error (ppm)	Formula	Estimated structure
315.23255	2.21	C21H31O2	
193.12270	2.05	C12H17O2	
259.16952	1.00	C17H23O2	
333.24309	2.00	C21H33O3	

Table 4: The elemental composition, the estimated structure, the observed m/z with the mass intensive, but unspecific high energy fragments generated from [HHC-H2+O2] metabolites (M07, M08, M09, M10, M11 and M12)

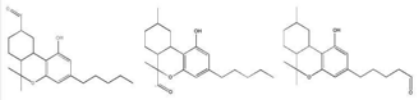
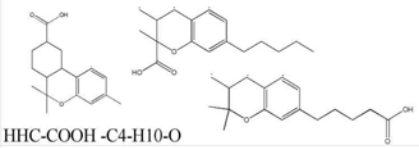
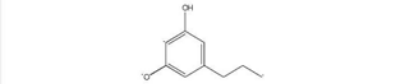
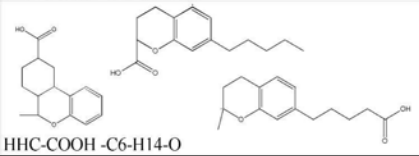
Observed m/z	Formula	Estimated structure
329.21112	C21H29O3	 HHC-COOH -O-H2
273.14852	C17H21O3	 HHC-COOH -C4-H10-O
149.05971	C9H9O2	 HHC-COOH -C12-H22-O2
247.13287	C15H19O3	 HHC-COOH -C6-H14-O

Table 5: The elemental composition, the estimated structure, the observed m/z with the mass error in ppm compared to the expected m/z of the 21 most intensive high energy fragments generated from M13

[HHC+2(-H2+O2)] metabolite, Rt: 11,48 min

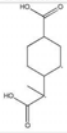
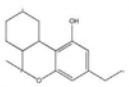
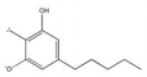
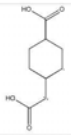
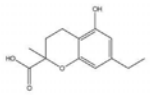
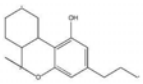
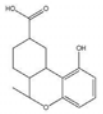
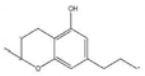
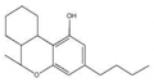
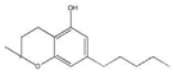
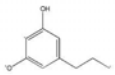
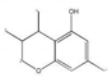
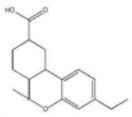
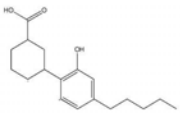
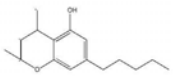
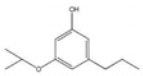
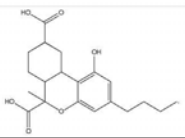
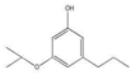
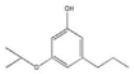
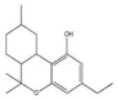
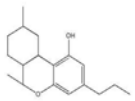
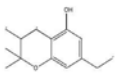
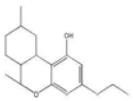
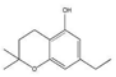
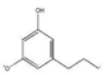
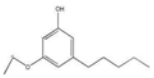
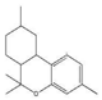
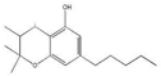
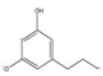
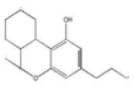
Observed m/z	Mass error (ppm)	Formula	Estimated structure	Observed m/z	Mass error (ppm)	Formula	Estimated structure
197,08206	6	C10H13O4		243,13945	6,14	C16H19O2	
191,10654	-0,63	C12H15O2		217,1225	0,87	C14H17O2	
184,07437	7,15	C9H12O4		237,11257	1,76	C13H17O4	
257,15419	2,29	C17H21O2		263,12906	4,75	C15H19O4	
201,09195	4,69	C13H13O2		203,10784	5,83	C13H15O2	
271,16952	0,97	C18H23O2		233,15479	5,07	C15H21O2	
149,06012	2,77	C9H9O2		175,07552	0,93	C11H11O2	
187,0754	0,23	C12H11O2		273,14877	0,82	C17H21O3	
287,16396	-0,82	C18H23O3		245,15527	6,77	C16H21O2	
193,12219	-0,59	C12H17O2		362,17214	-0,82	C20H26O6	

Table 6: The elemental composition, the estimated structure, the observed m/z with the mass error in ppm compared to the expected m/z of the 12 most intensive high energy fragments generated from the two HHC epimers(left column: (9S)-HHC; right column: (9R)-HHC)

Observed m/z	Mass error (ppm)	Formula	Estimated structure	Observed m/z	Mass error (ppm)	Formula	Estimated structure
193,12282	2,68	C12H17O2		193,12294	3,27	C12H17O2	
273,18533	1,56	C18H25O2		271,17014	3,26	C18H23O2	
123,0447	5,21	C7H7O2		123,04472	5,38	C7H7O2	
137,13282	2,52	C10H17		215,10755	4,14	C14H15O2	
137,06011	2,98	C8H9O2		137,06015	3,26	C8H9O2	
271,16975	1,82	C18H23O2		201,09166	3,24	C13H13O2	
207,13805	0,47	C13H19O2		149,06029	3,92	C9H9O2	
205,12264	1,64	C13H17O2		243,17382	-2,13	C17H23O	
261,18308	-7,01	C17H25O2		109,06524	4,14	C7H9O	
149,05997	1,74	C9H9O2		257,15438	2,99	C17H21O2	
163,07537	0,07	C10H11O2		137,13265	1,25	C10H17	
109,06509	2,71	C7H9O		163,07539	0,2	C10H11O2	

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We accept the manuscript submissions in any standard (generic) format.

We typeset manuscripts using advanced typesetting tools like Adobe In Design, CorelDraw, TeXnicCenter, and TeXStudio. We usually recommend authors submit their research using any standard format they are comfortable with, and let Global Journals do the rest.

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Acknowledgments

Contributors to the research other than authors credited should be mentioned in Acknowledgments. The source of funding for the research can be included. Suppliers of resources may be mentioned along with their addresses.

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The following is the official style and template developed for publication of a research paper. Authors are not required to follow this style during the submission of the paper. It is just for reference purposes.

Manuscript Style Instruction (Optional)

- Microsoft Word Document Setting Instructions.
- Font type of all text should be Swis721 Lt BT.
- Page size: 8.27" x 11'", left margin: 0.65, right margin: 0.65, bottom margin: 0.75.
- Paper title should be in one column of font size 24.
- Author name in font size of 11 in one column.
- Abstract: font size 9 with the word "Abstract" in bold italics.
- Main text: font size 10 with two justified columns.
- Two columns with equal column width of 3.38 and spacing of 0.2.
- First character must be three lines drop-capped.
- The paragraph before spacing of 1 pt and after of 0 pt.
- Line spacing of 1 pt.
- Large images must be in one column.
- The names of first main headings (Heading 1) must be in Roman font, capital letters, and font size of 10.
- The names of second main headings (Heading 2) must not include numbers and must be in italics with a font size of 10.

Structure and Format of Manuscript

The recommended size of an original research paper is under 15,000 words and review papers under 7,000 words. Research articles should be less than 10,000 words. Research papers are usually longer than review papers. Review papers are reports of significant research (typically less than 7,000 words, including tables, figures, and references)

A research paper must include:

a) A title which should be relevant to the theme of the paper.
b) A summary, known as an abstract (less than 150 words), containing the major results and conclusions.
c) Up to 10 keywords that precisely identify the paper's subject, purpose, and focus.
d) An introduction, giving fundamental background objectives.
e) Resources and techniques with sufficient complete experimental details (wherever possible by reference) to permit repetition, sources of information must be given, and numerical methods must be specified by reference.
f) Results which should be presented concisely by well-designed tables and figures.
g) Suitable statistical data should also be given.
h) All data must have been gathered with attention to numerical detail in the planning stage.

Design has been recognized to be essential to experiments for a considerable time, and the editor has decided that any paper that appears not to have adequate numerical treatments of the data will be returned unrefereed.

i) Discussion should cover implications and consequences and not just recapitulate the results; conclusions should also be summarized.
j) There should be brief acknowledgments.
k) There ought to be references in the conventional format. Global Journals recommends APA format.

Authors should carefully consider the preparation of papers to ensure that they communicate effectively. Papers are much more likely to be accepted if they are carefully designed and laid out, contain few or no errors, are summarizing, and follow instructions. They will also be published with much fewer delays than those that require much technical and editorial correction.

The Editorial Board reserves the right to make literary corrections and suggestions to improve brevity.

Format Structure

It is necessary that authors take care in submitting a manuscript that is written in simple language and adheres to published guidelines.

All manuscripts submitted to Global Journals should include:

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The title page must carry an informative title that reflects the content, a running title (less than 45 characters together with spaces), names of the authors and co-authors, and the place(s) where the work was carried out.

Author details

The full postal address of any related author(s) must be specified.

Abstract

The abstract is the foundation of the research paper. It should be clear and concise and must contain the objective of the paper and inferences drawn. It is advised to not include big mathematical equations or complicated jargon.

Many researchers searching for information online will use search engines such as Google, Yahoo or others. By optimizing your paper for search engines, you will amplify the chance of someone finding it. In turn, this will make it more likely to be viewed and cited in further works. Global Journals has compiled these guidelines to facilitate you to maximize the web-friendliness of the most public part of your paper.

Keywords

A major lynchpin of research work for the writing of research papers is the keyword search, which one will employ to find both library and internet resources. Up to eleven keywords or very brief phrases have to be given to help data retrieval, mining, and indexing.

One must be persistent and creative in using keywords. An effective keyword search requires a strategy: planning of a list of possible keywords and phrases to try.

Choice of the main keywords is the first tool of writing a research paper. Research paper writing is an art. Keyword search should be as strategic as possible.

One should start brainstorming lists of potential keywords before even beginning searching. Think about the most important concepts related to research work. Ask, “What words would a source have to include to be truly valuable in a research paper?” Then consider synonyms for the important words.

It may take the discovery of only one important paper to steer in the right keyword direction because, in most databases, the keywords under which a research paper is abstracted are listed with the paper.

Numerical Methods

Numerical methods used should be transparent and, where appropriate, supported by references.

Abbreviations

Authors must list all the abbreviations used in the paper at the end of the paper or in a separate table before using them.

Formulas and equations

Authors are advised to submit any mathematical equation using either MathJax, KaTeX, or LaTeX, or in a very high-quality image.

Tables, Figures, and Figure Legends

Tables: Tables should be cautiously designed, uncrowned, and include only essential data. Each must have an Arabic number, e.g., Table 4, a self-explanatory caption, and be on a separate sheet. Authors must submit tables in an editable format and not as images. References to these tables (if any) must be mentioned accurately.

Figures

Figures are supposed to be submitted as separate files. Always include a citation in the text for each figure using Arabic numbers, e.g., Fig. 4. Artwork must be submitted online in vector electronic form or by emailing it.

Preparation of Eletronic Figures for Publication

Although low-quality images are sufficient for review purposes, print publication requires high-quality images to prevent the final product being blurred or fuzzy. Submit (possibly by e-mail) EPS (line art) or TIFF (halftone/ photographs) files only. MS PowerPoint and Word Graphics are unsuitable for printed pictures. Avoid using pixel-oriented software. Scans (TIFF only) should have a resolution of at least 350 dpi (halftone) or 700 to 1100 dpi (line drawings). Please give the data for figures in black and white or submit a Color Work Agreement form. EPS files must be saved with fonts embedded (and with a TIFF preview, if possible).

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Tips for writing a good quality Medical Research Paper

1. Choosing the topic: In most cases, the topic is selected by the interests of the author, but it can also be suggested by the guides. You can have several topics, and then judge which you are most comfortable with. This may be done by asking several questions of yourself, like "Will I be able to carry out a search in this area? Will I find all necessary resources to accomplish the search? Will I be able to find all information in this field area?" If the answer to this type of question is "yes," then you ought to choose that topic. In most cases, you may have to conduct surveys and visit several places. Also, you might have to do a lot of work to find all the rises and falls of the various data on that subject. Sometimes, detailed information plays a vital role, instead of short information. Evaluators are human: The first thing to remember is that evaluators are also human beings. They are not only meant for rejecting a paper. They are here to evaluate your paper. So present your best aspect.

2. Think like evaluators: If you are in confusion or getting demotivated because your paper may not be accepted by the evaluators, then think, and try to evaluate your paper like an evaluator. Try to understand what an evaluator wants in your research paper, and you will automatically have your answer. Make blueprints of paper: The outline is the plan or framework that will help you to arrange your thoughts. It will make your paper logical. But remember that all points of your outline must be related to the topic you have chosen.

3. Ask your guides: If you are having any difficulty with your research, then do not hesitate to share your difficulty with your guide (if you have one). They will surely help you out and resolve your doubts. If you can't clarify what exactly you require for your work, then ask your supervisor to help you with an alternative. He or she might also provide you with a list of essential readings.

4. Use of computer is recommended: As you are doing research in the field of medical research then this point is quite obvious. Use right software: Always use good quality software packages. If you are not capable of judging good software, then you can lose the quality of your paper unknowingly. There are various programs available to help you which you can get through the internet.

5. Use the internet for help: An excellent start for your paper is using Google. It is a wondrous search engine, where you can have your doubts resolved. You may also read some answers for the frequent question of how to write your research paper or find a model research paper. You can download books from the internet. If you have all the required books, place importance on reading, selecting, and analyzing the specified information. Then sketch out your research paper. Use big pictures: You may use encyclopedias like Wikipedia to get pictures with the best resolution. At Global Journals, you should strictly follow here.

6. Bookmarks are useful: When you read any book or magazine, you generally use bookmarks, right? It is a good habit which helps to not lose your continuity. You should always use bookmarks while searching on the internet also, which will make your search easier.

7. Revise what you wrote: When you write anything, always read it, summarize it, and then finalize it.

8. Make every effort: Make every effort to mention what you are going to write in your paper. That means always have a good start. Try to mention everything in the introduction—what is the need for a particular research paper. Polish your work with good writing skills and always give an evaluator what he wants. Make backups: When you are going to do any important thing like making a research paper, you should always have backup copies of it either on your computer or on paper. This protects you from losing any portion of your important data.

9. Produce good diagrams of your own: Always try to include good charts or diagrams in your paper to improve quality. Using several unnecessary diagrams will degrade the quality of your paper by creating a hodgepodge. So always try to include diagrams which were made by you to improve the readability of your paper. Use of direct quotes: When you do research relevant to literature, history, or current affairs, then use of quotes becomes essential, but if the study is relevant to science, use of quotes is not preferable.

10. Use proper verb tense: Use proper verb tenses in your paper. Use past tense to present those events that have happened. Use present tense to indicate events that are going on. Use future tense to indicate events that will happen in the future. Use of wrong tenses will confuse the evaluator. Avoid sentences that are incomplete.

11. Pick a good study spot: Always try to pick a spot for your research which is quiet. Not every spot is good for studying.

12. Know what you know: Always try to know what you know by making objectives, otherwise you will be confused and unable to achieve your target.

13. Use good grammar: Always use good grammar and words that will have a positive impact on the evaluator; use of good vocabulary does not mean using tough words which the evaluator has to find in a dictionary. Do not fragment sentences. Eliminate one-word sentences. Do not ever use a big word when a smaller one would suffice.

Verbs have to be in agreement with their subjects. In a research paper, do not start sentences with conjunctions or finish them with prepositions. When writing formally, it is advisable to never split an infinitive because someone will (wrongly) complain. Avoid clichés like a disease. Always shun irritating alliteration. Use language which is simple and straightforward. Put together a neat summary.

14. Arrangement of information: Each section of the main body should start with an opening sentence, and there should be a changeover at the end of the section. Give only valid and powerful arguments for your topic. You may also maintain your arguments with records.

15. Never start at the last minute: Always allow enough time for research work. Leaving everything to the last minute will degrade your paper and spoil your work.

16. Multitasking in research is not good: Doing several things at the same time is a bad habit in the case of research activity. Research is an area where everything has a particular time slot. Divide your research work into parts, and do a particular part in a particular time slot.

17. Never copy others' work: Never copy others' work and give it your name because if the evaluator has seen it anywhere, you will be in trouble. Take proper rest and food: No matter how many hours you spend on your research activity, if you are not taking care of your health, then all your efforts will have been in vain. For quality research, take proper rest and food.

18. Go to seminars: Attend seminars if the topic is relevant to your research area. Utilize all your resources.

19. Refresh your mind after intervals: Try to give your mind a rest by listening to soft music or sleeping in intervals. This will also improve your memory. Acquire colleagues: Always try to acquire colleagues. No matter how sharp you are, if you acquire colleagues, they can give you ideas which will be helpful to your research.

20. Think technically: Always think technically. If anything happens, search for its reasons, benefits, and demerits. Think and then print: When you go to print your paper, check that tables are not split, headings are not detached from their descriptions, and page sequence is maintained.

21. Adding unnecessary information: Do not add unnecessary information like "I have used MS Excel to draw graphs." Irrelevant and inappropriate material is superfluous. Foreign terminology and phrases are not apropos. One should never take a broad view. Analogy is like feathers on a snake. Use words properly, regardless of how others use them. Remove quotations. Puns are for kids, not grunt readers. Never oversimplify: When adding material to your research paper, never go for oversimplification; this will definitely irritate the evaluator. Be specific. Never use rhythmic redundancies. Contractions shouldn't be used in a research paper. Comparisons are as terrible as clichés. Give up ampersands, abbreviations, and so on. Remove commas that are not necessary. Parenthetical words should be between brackets or commas. Understatement is always the best way to put forward earth-shaking thoughts. Give a detailed literary review.

22. Report concluded results: Use concluded results. From raw data, filter the results, and then conclude your studies based on measurements and observations taken. An appropriate number of decimal places should be used. Parenthetical remarks are prohibited here. Proofread carefully at the final stage. At the end, give an outline to your arguments. Spot perspectives of further study of the subject. Justify your conclusion at the bottom sufficiently, which will probably include examples.

23. Upon conclusion: Once you have concluded your research, the next most important step is to present your findings. Presentation is extremely important as it is the definite medium though which your research is going to be in print for the rest of the crowd. Care should be taken to categorize your thoughts well and present them in a logical and neat manner. A good quality research paper format is essential because it serves to highlight your research paper and bring to light all necessary aspects of your research.

Informal Guidelines of Research Paper Writing

Key points to remember:

- Submit all work in its final form.
- Write your paper in the form which is presented in the guidelines using the template.
- Please note the criteria peer reviewers will use for grading the final paper.

Final points:

One purpose of organizing a research paper is to let people interpret your efforts selectively. The journal requires the following sections, submitted in the order listed, with each section starting on a new page:

The introduction: This will be compiled from reference matter and reflect the design processes or outline of basis that directed you to make a study. As you carry out the process of study, the method and process section will be constructed like that. The results segment will show related statistics in nearly sequential order and direct reviewers to similar intellectual paths throughout the data that you gathered to carry out your study.

The discussion section:

This will provide understanding of the data and projections as to the implications of the results. The use of good quality references throughout the paper will give the effort trustworthiness by representing an alertness to prior workings.

Writing a research paper is not an easy job, no matter how trouble-free the actual research or concept. Practice, excellent preparation, and controlled record-keeping are the only means to make straightforward progression.

General style:

Specific editorial column necessities for compliance of a manuscript will always take over from directions in these general guidelines.

To make a paper clear: Adhere to recommended page limits.

Mistakes to avoid:

- Insertion of a title at the foot of a page with subsequent text on the next page.
- Separating a table, chart, or figure—confine each to a single page.
- Submitting a manuscript with pages out of sequence.
- In every section of your document, use standard writing style, including articles ("a" and "the").
- Keep paying attention to the topic of the paper.
- Use paragraphs to split each significant point (excluding the abstract).
- Align the primary line of each section.
- Present your points in sound order.
- Use present tense to report well-accepted matters.
- Use past tense to describe specific results.
- Do not use familiar wording; don't address the reviewer directly. Don't use slang or superlatives.
- Avoid use of extra pictures—include only those figures essential to presenting results.

Title page:

Choose a revealing title. It should be short and include the name(s) and address(es) of all authors. It should not have acronyms or abbreviations or exceed two printed lines.

Abstract: This summary should be two hundred words or less. It should clearly and briefly explain the key findings reported in the manuscript and must have precise statistics. It should not have acronyms or abbreviations. It should be logical in itself. Do not cite references at this point.

An abstract is a brief, distinct paragraph summary of finished work or work in development. In a minute or less, a reviewer can be taught the foundation behind the study, common approaches to the problem, relevant results, and significant conclusions or new questions.

Write your summary when your paper is completed because how can you write the summary of anything which is not yet written? Wealth of terminology is very essential in abstract. Use comprehensive sentences, and do not sacrifice readability for brevity; you can maintain it succinctly by phrasing sentences so that they provide more than a lone rationale. The author can at this moment go straight to shortening the outcome. Sum up the study with the subsequent elements in any summary. Try to limit the initial two items to no more than one line each.

Reason for writing the article—theory, overall issue, purpose.

- Fundamental goal.
- To-the-point depiction of the research.
- Consequences, including definite statistics—if the consequences are quantitative in nature, account for this; results of any numerical analysis should be reported. Significant conclusions or questions that emerge from the research.

Approach:

- Single section and succinct.
- An outline of the job done is always written in past tense.
- Concentrate on shortening results—limit background information to a verdict or two.
- Exact spelling, clarity of sentences and phrases, and appropriate reporting of quantities (proper units, important statistics) are just as significant in an abstract as they are anywhere else.

Introduction:

The introduction should "introduce" the manuscript. The reviewer should be presented with sufficient background information to be capable of comprehending and calculating the purpose of your study without having to refer to other works. The basis for the study should be offered. Give the most important references, but avoid making a comprehensive appraisal of the topic. Describe the problem visibly. If the problem is not acknowledged in a logical, reasonable way, the reviewer will give no attention to your results. Speak in common terms about techniques used to explain the problem, if needed, but do not present any particulars about the protocols here.

The following approach can create a valuable beginning:

- Explain the value (significance) of the study.
- Defend the model—why did you employ this particular system or method? What is its compensation? Remark upon its appropriateness from an abstract point of view as well as pointing out sensible reasons for using it.
- Present a justification. State your particular theory(-ies) or aim(s), and describe the logic that led you to choose them.
- Briefly explain the study's tentative purpose and how it meets the declared objectives.

Approach:

Use past tense except for when referring to recognized facts. After all, the manuscript will be submitted after the entire job is done. Sort out your thoughts; manufacture one key point for every section. If you make the four points listed above, you will need at least four paragraphs. Present surrounding information only when it is necessary to support a situation. The reviewer does not desire to read everything you know about a topic. Shape the theory specifically—do not take a broad view.

As always, give awareness to spelling, simplicity, and correctness of sentences and phrases.

Procedures (methods and materials):

This part is supposed to be the easiest to carve if you have good skills. A soundly written procedures segment allows a capable scientist to replicate your results. Present precise information about your supplies. The suppliers and clarity of reagents can be helpful bits of information. Present methods in sequential order, but linked methodologies can be grouped as a segment. Be concise when relating the protocols. Attempt to give the least amount of information that would permit another capable scientist to replicate your outcome, but be cautious that vital information is integrated. The use of subheadings is suggested and ought to be synchronized with the results section.

When a technique is used that has been well-described in another section, mention the specific item describing the way, but draw the basic principle while stating the situation. The purpose is to show all particular resources and broad procedures so that another person may use some or all of the methods in one more study or referee the scientific value of your work. It is not to be a step-by-step report of the whole thing you did, nor is a methods section a set of orders.

Materials:

Materials may be reported in part of a section or else they may be recognized along with your measures.

Methods:

- Report the method and not the particulars of each process that engaged the same methodology.
- Describe the method entirely.
- To be succinct, present methods under headings dedicated to specific dealings or groups of measures.
- Simplify—detail how procedures were completed, not how they were performed on a particular day.
- If well-known procedures were used, account for the procedure by name, possibly with a reference, and that's all.

Approach:

It is embarrassing to use vigorous voice when documenting methods without using first person, which would focus the reviewer's interest on the researcher rather than the job. As a result, when writing up the methods, most authors use third person passive voice.

Use standard style in this and every other part of the paper—avoid familiar lists, and use full sentences.

What to keep away from:

- Resources and methods are not a set of information.
- Skip all descriptive information and surroundings—save it for the argument.
- Leave out information that is immaterial to a third party.

Results:

The principle of a results segment is to present and demonstrate your conclusion. Create this part as entirely objective details of the outcome, and save all understanding for the discussion.

The page length of this segment is set by the sum and types of data to be reported. Use statistics and tables, if suitable, to present consequences most efficiently.

You must clearly differentiate material which would usually be incorporated in a study editorial from any unprocessed data or additional appendix matter that would not be available. In fact, such matters should not be submitted at all except if requested by the instructor.

Content:

- Sum up your conclusions in text and demonstrate them, if suitable, with figures and tables.
- In the manuscript, explain each of your consequences, and point the reader to remarks that are most appropriate.
- Present a background, such as by describing the question that was addressed by creation of an exacting study.
- Explain results of control experiments and give remarks that are not accessible in a prescribed figure or table, if appropriate.
- Examine your data, then prepare the analyzed (transformed) data in the form of a figure (graph), table, or manuscript.

What to stay away from:

- Do not discuss or infer your outcome, report surrounding information, or try to explain anything.
- Do not include raw data or intermediate calculations in a research manuscript.
- Do not present similar data more than once.
- A manuscript should complement any figures or tables, not duplicate information.
- Never confuse figures with tables—there is a difference.

Approach:

As always, use past tense when you submit your results, and put the whole thing in a reasonable order.

Put figures and tables, appropriately numbered, in order at the end of the report.

If you desire, you may place your figures and tables properly within the text of your results section.

Figures and tables:

If you put figures and tables at the end of some details, make certain that they are visibly distinguished from any attached appendix materials, such as raw facts. Whatever the position, each table must be titled, numbered one after the other, and include a heading. All figures and tables must be divided from the text.

Discussion:

The discussion is expected to be the trickiest segment to write. A lot of papers submitted to the journal are discarded based on problems with the discussion. There is no rule for how long an argument should be.

Position your understanding of the outcome visibly to lead the reviewer through your conclusions, and then finish the paper with a summing up of the implications of the study. The purpose here is to offer an understanding of your results and support all of your conclusions, using facts from your research and generally accepted information, if suitable. The implication of results should be fully described.

Infer your data in the conversation in suitable depth. This means that when you clarify an observable fact, you must explain mechanisms that may account for the observation. If your results vary from your prospect, make clear why that may have happened. If your results agree, then explain the theory that the proof supported. It is never suitable to just state that the data approved the prospect, and let it drop at that. Make a decision as to whether each premise is supported or discarded or if you cannot make a conclusion with assurance. Do not just dismiss a study or part of a study as "uncertain."

Research papers are not acknowledged if the work is imperfect. Draw what conclusions you can based upon the results that you have, and take care of the study as a finished work.

- You may propose future guidelines, such as how an experiment might be personalized to accomplish a new idea.
- Give details of all of your remarks as much as possible, focusing on mechanisms.
- Make a decision as to whether the tentative design sufficiently addressed the theory and whether or not it was correctly restricted. Try to present substitute explanations if they are sensible alternatives.
- One piece of research will not counter an overall question, so maintain the large picture in mind. Where do you go next? The best studies unlock new avenues of study. What questions remain?
- Recommendations for detailed papers will offer supplementary suggestions.

Approach:

When you refer to information, differentiate data generated by your own studies from other available information. Present work done by specific persons (including you) in past tense.

Describe generally acknowledged facts and main beliefs in present tense.

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	A-B	**C-D**	**E-F**
Abstract	Clear and concise with appropriate content, Correct format. 200 words or below	Unclear summary and no specific data, Incorrect form Above 200 words	No specific data with ambiguous information Above 250 words
Introduction	Containing all background details with clear goal and appropriate details, flow specification, no grammar and spelling mistake, well organized sentence and paragraph, reference cited	Unclear and confusing data, appropriate format, grammar and spelling errors with unorganized matter	Out of place depth and content, hazy format
Methods and Procedures	Clear and to the point with well arranged paragraph, precision and accuracy of facts and figures, well organized subheads	Difficult to comprehend with embarrassed text, too much explanation but completed	Incorrect and unorganized structure with hazy meaning
Result	Well organized, Clear and specific, Correct units with precision, correct data, well structuring of paragraph, no grammar and spelling mistake	Complete and embarrassed text, difficult to comprehend	Irregular format with wrong facts and figures
Discussion	Well organized, meaningful specification, sound conclusion, logical and concise explanation, highly structured paragraph reference cited	Wordy, unclear conclusion, spurious	Conclusion is not cited, unorganized, difficult to comprehend
References	Complete and correct format, well organized	Beside the point, Incomplete	Wrong format and structuring

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