Journal of HyoJeong Academia

Article

Insight into the therapeutic potential of Antarctic mosses: An untargeted metabolomics approach

Kristina Lama [1,2], Ryoichi Yamada [1,2], Hirotake Yamaguchi [1,2] and Tae-Jin Oh [1,2,3]*

[1] Department of Life Science and Biochemical Engineering, Sun Moon University, Asan, 31460, Korea
[2] Bio Bigdata-based Chungnam Smart Clean Research Leader Training Program, Sun Moon University
[3] Genome-based BioIT Convergence Institute, Sun Moon University
* Correspondence: tjoh3782@sunmoon.ac.kr

Abstract: Over the course of history, plant materials have been utilized for healing various ailments. The knowledge derived from traditional medicine has proven invaluable, serving as a foundational resource for drug discovery and design. However, the journey from screening natural products to the development of drugs is a prolonged and demanding process. Fortunately, with the emergence of sophisticated analytical techniques and the establishment of global databases containing information on thousands of natural products, the screening and discovery of bioactive compounds from complex sample mixtures have become more time-efficient and less labor-intensive. In particular, the non-targeted metabolomics approach proves advantageous, offering a comprehensive analysis of a wide range of compounds, aiding in the detection of both known and unknown substances. This approach is especially beneficial for gaining a thorough understanding of the pattern of metabolites found in Arctic and Antarctic vegetation, among the least explored areas on Earth. Here, we employed a non-targeted metabolomics approach to analyze the chemical components in Antarctic mosses. While many of the compounds could not be predicted, some were identified as therapeutic agents, with a few exhibiting pharmacological properties such as anticancer, anti-inflammatory, antilipemic, anti-diabetic, anti-adepogenic, and neuroprotective effects. Notably, we predicted that one moss sample produced desulfiram, an FDA-approved drug for the treatment of chronic alcoholism, and methyl palmitate, well-known for its anti-inflammatory potency, could be detected in many of the samples. Furthermore, we experimentally verified the therapeutic potential of Antarctic moss extracts by conducting a cyclooxygenase-2 (COX-2) inhibition assay, assessing their potent anti-inflammatory activity. Indeed, two of the moss samples exhibited significant COX-2 inhibitory potency. Hence, our findings emphasize the advantage of metabolomics in providing insight into the potential of Antarctic moss as a valuable natural resource for the discovery and development of potent therapeutics. Additional research is due in order to identify and define the particular bioactive compounds accountable for the observed effects.

Keywords: Antarctic mosses, non-targeted metabolomics, metabolites, pharmacological properties, COX-2 inhibition.

Citation: Kristina Lama, Ryoichi Yamada, Hirotake Yamaguchi and Tae-Jin Oh. Insight into the therapeutic potential of Antarctic mosses: An untargeted metabolomics approach. *JHJA* 2(2), **2024**.

Received: 11 March 2024
Accepted: 23 May 2024
Published: 30 July 2024

1. Introduction

Indigenous knowledge, traditional medicine, and ethnopharmacology have forged a rich foundation for the discovery of therapeutic agents [1]. The wealth of information embedded in the wisdom of diverse cultures and centuries-old practices, often rooted in the use of local flora for medicinal purposes, has set the stage for unlocking the latent potential of nature's pharmacy [2, 3]. Even though the use of natural products (NPs) for medicinal purposes dates back to 2600 BC [4], the chemical space of undiscovered NPs still outweighs that of those already known [5, 6]. This setback could be due to high cost, time consuming and labor-intensive applied methods [7]. However, recent advancements in

computing and information processing keep us hopeful as we can systematically explore uncharted territories for potential drug candidates by harnessing the power of high-throughput screening processes [8]. The rapid analysis of vast datasets across available databases of natural products will expedite the identification of promising compounds from untapped resources [9].

Metabolomics refers to the comprehensive measurement of all metabolites and low-molecular-weight molecules in a biological specimen. And metabolic profiling is the systematic analysis, identification, and quantification of metabolites within a specific biological system [10]. Both processes are valuable for understanding the complex metabolic processes and their respective metabolites. Based on the approach two types of metabolomics can be applied, targeted and non-targeted. As the name suggests, non-targeted metabolomics encompasses the global measurement of all metabolites in a sample, including both known and unknown targets [11]. Utilizing analytical techniques such as mass spectrometry, non-targeted metabolomics offers high-throughput screening of compounds [12].

For example, untargeted metabolomics has been extensively used in the study of traditional Chinese medicine (TCM) for purposes ranging from identifying bioactive compounds [13] to evaluating toxicology [14]. Additionally, by integrating metabolomics with network pharmacology, researchers have proposed optimized formulations of TCM for treating various systemic diseases [15, 16]. These examples clearly demonstrate that untargeted metabolomics is a key trend in natural product research, drug discovery, and development.

Integrating metabolomics for the exploration of Antarctic mosses highlights the importance of this captivating frontier in drug discovery. These resilient organisms, thriving in one of the harshest environments on Earth, hold the promise of unique bioactive compounds (anti-freeze proteins, UV-protective substances, or novel antibiotics) [17]. By applying an untargeted metabolomics approach, we can delve into the intricate and unique biochemistry of Antarctic mosses, unraveling their therapeutic potential. The vast natural product database, cultivated through the amalgamation of traditional knowledge and computational screening, serves as a reservoir of information to guide this exploration.

Therefore, this study carried out the exploration of the less studied resource Antarctic mosses leveraging analytical techniques, computational tools and database searching (metabolomics) to unravel their therapeutic potential. Furthermore, experimental evaluation was performed where the moss extracts were assessed for their bioactivity using COX-2, an enzyme involved in inflammatory response in the body [18]. The significance of the COX-2 inhibition assay lies in its ability to identify compounds that can potentially act as anti-inflammatory agents, which is crucial for developing new treatments for inflammatory diseases.

2. Materials and Methods

2.1. Chemicals

Solvents used for extraction such as methanol and acetone were purchased from Daejung Reagents Chemicals (>99.8%) and Samchun Pure Chemicals (99.7%) respectively. HPLC grade solvents were used for Ultra-High Performance Liqiuid Chromatography (UHPLC) bought from Fisher Scientific, Korea. A COX ovine/human Inhibitor Screening Assay Kit 560131 (Cayman Chemical, Ann Arbor, MI, USA) was used.

2.2. Moss sample processing and extraction

Moss samples were obtained from the Polar Natural Product Chemistry Laboratory of the Korea Polar Research Institute, here labeled as MS-1, MS-3, MS-6, MS-9, MS-10, and MS-13. Each samples were extracted using two organic solvents: acetone and methanol. Processing involved grounding of the dried samples in a mortar, weighing about 3 grams of the powdered sample and distribution into each flasks. The flasks were filled with (200 mL) acetone and extracted at dark and room temperature condition for 24 hours with constant stirring. The mixture was filtered, and the filtrate extracts dried under reduced pressure. The process was repeated thrice and after the third extraction, the solvent was replaced by methanol and the process continued as before. In this way, acetone and methanol extracts for each moss samples were obtained. The dried crude extracts were dissolved in HPLC grade methanol for futher analysis.

2.3. Untargeted metabolomics

2.3.1.UHPLC analysis

Preliminary analysis of metabolites was conducted on a Shimadzu Nexera UHPLC system equipped with a Shim-pack GIS C18 column (4.6 X 250 mm, particle size 5 μm HSS) connected to a Photo Diode Array (PDA) Detector. The mobile phase consisted of two solvent system, solvent A consituting of water and solvent B, acetonitrile. The elution method followed a gradient flow starting off at 5% solvent B reaching 100% at 28 min. The flow rate was maintained at 1.0 mL/min.

2.3.2. LC-MS/MS analysis

A Thermo Scientific UHPLC, Ultimate 3,000 RSLC System coupled to a Q-Exactive Plus Orbitrap mass spectrometer was used for untargeted LC-MS/MS. Chromatographic separation in the LC system carried out using Acquity UPLC BEH C18 column (2.1 X 100 mm, 1.7 μm) with two solvents water (C) and acetonitrile (D), both acidified with 0.1% formic acid. The gradient elution method was as follows: 5% D (2 min), 5-100% D (2-9min), 100% D (9-13min), 100-5% D (13-13.1 min), and 5% D (13-16min). the column temperature was maintained at 50°C, injection volume of 5 μL and flow rate of 0.4 mL/min. Mass spectra were recorded in full MS-ddMS2 positive mode at the range of 80-1000 m/z. The operation parameters were as follows: collision-induced dissociation energy, 30 V; resolution, 70,000 for full MS and 17500 for ddMS2; ion spray voltage, 3.5 kV; and capillary temperature, 370°C. The Raw MS data files were searched against databases such as High-Resolution Accurate Mass (HRAM), MassList, ChemSpider, etc. and matched spectrums annotated.

2.4. Raw data processing and molecular network building

Futher analysis of the raw data was performed as described previously [19,20] with some modifications. Briefly, the raw files were convert into a suitable "mzML" format. For molecular network building, Global Natural Product Social (GNPS) (https://gnps.ucsd.edu/ProteoSAFe/static/gnps-splash.jsp) molecular networking platform was utilized. The coverted files were uploaded into the GNPS site through WinSCP software. Another platform, SNAP-MS (www.npatlas.org/discover/snapms), processed the network generated from GNPS, annotating the subnetworks. The resulted files were downloaded as a graphML network file and visualized in Cytoscape (http://cytoscape.org/).

2.5. COX-2 inhibition asssay

In-vitro COX-2 inhibition assay was carried out using COX (ovine/human) Inhibitor Screening Assay kit from Cayman Chemical following the instruction in the manual provided. The tested concentrations were 50 and 500 μg/mL. Postive control contained only DMSO. For negative control, the enzyme was subjected to thermal denaturation. Data were reperesented as the percentage of control.

2.6. Statistical analysis

Experiments were performed in triplicate and the results were expressed in terms of mean ± standard deviation.

3. Results

3.1. Preliminary analysis of metabolites

Reversed phase HPLC of the crude extracts of Antarctic mosses showed multiple peaks in the chromatogram indicating the presence of diverse range of compounds with varying polarity. Figure 1 shows chromatogram of MS-10 and MS-13 acetone and methanol extracts with solvent blank as methanol. Indicated by red arrows, distinct peaks may be unique components of the extracts. In comparison to Ms-13 methanol and acetone extracts, MS-10 methanol extracts contained relatively polar compounds as the shown by the early elution time (rt < 6.0 minutes) of the peaks which indicated that moss samples contained distinct compounds with differing chemical properties.

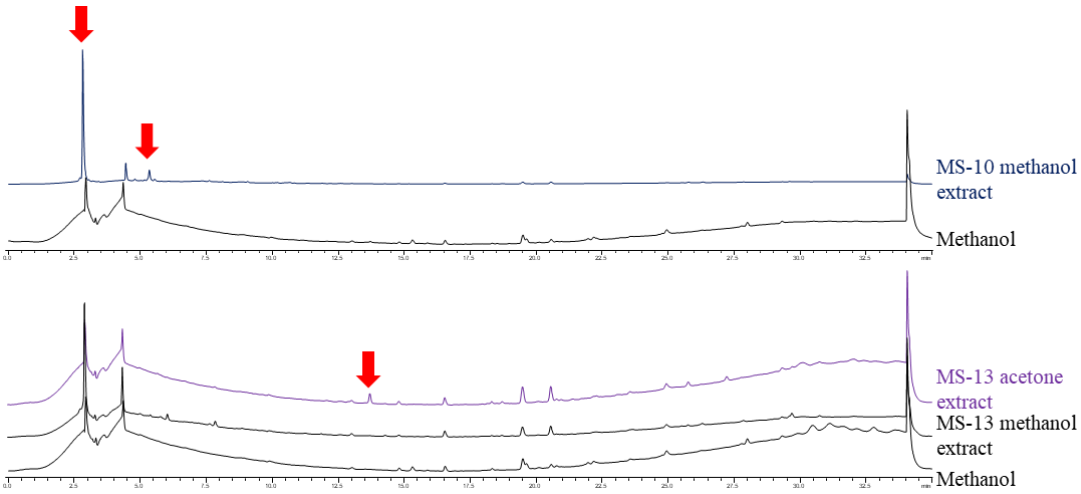


Figure 1. HPLC chromatogram of methanol and acetone extracts of MS-10 and MS-13 samples with distinct peaks indicated by red arrows.

3.2. Untargeted metabolomics

3.2.1. LC-MS/MS analysis

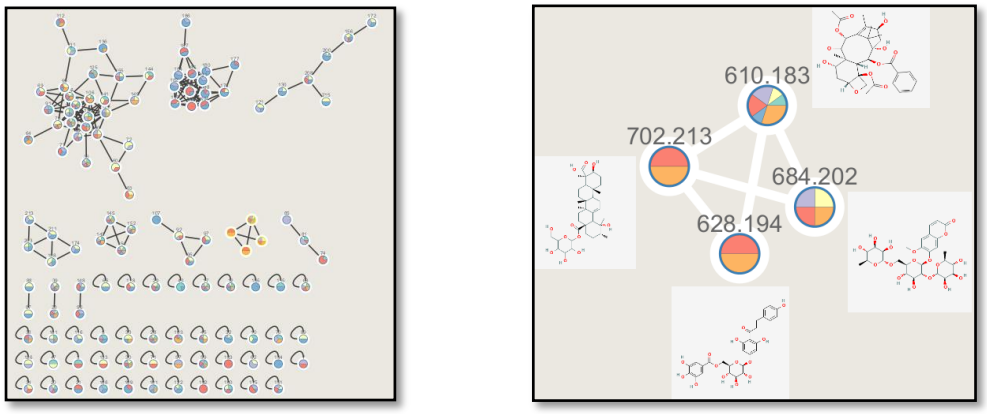
For a more comprehensive exploration of the metabolic profiles of the crude extracts, LC-MS/MS was performed. Database searching of the obtained MS/MS data aided in compound annotation. Indeed, some of the predicted compounds were known for their therapeutic properties, whereas few were FDA approved drugs. For example, disulfiram and meglutol were among the predicted compounds that are prescribed for treatment of alcohol addiction and as an antilipemic agent respectively. Table 1 lists the common annotated compounds predicted from MS-10 and MS-13 with their bioactivity.

Table 1. List of some compounds present in Antarctic mosses crude extracts and their features identified using LC-MS/MS analysis including their therapeutic properties (reference studies)

S. N.	Compound name	RT (min)	Molecular formula	Exact mass	Therapeutic potential
1	Choline alfoscerate	0.669	$C_8H_{20}NO_6P$	257.1023	Neuroprotective agent [21, 22]
2	Disulfiram	0.624	$C_{10}H_{20}N_2S_4$	296.0501	Treatment of alcohol use disorder [23]
3	D-Raffinose	0.594	$C_{18}H_{32}O_{16}$	504.1683	Anti-adipogenesis, anti-diabetic [24]
4	Meglutol	0.604	$C_6H_{10}O_5$	162.0526	HMG-CoA inhibitor, antilipemic agent [25]
5	Methyl palmitate	9.781	$C_{17}H_{34}O_2$	287.2818	Anti-inflammatory, anti-fibrotic effect [26]
6	Oleamide	9.813	$C_{18}H_{35}NO$	281.2713	Treatment of sleep disorder, neuropharmacological activity [27, 28]
7	Stigmasterol	12.986	$C_{29}H_{48}O$	412.3697	Anti-diabetic, anti-cancer [29, 30]

3.2.2. Molecular networking analysis

Molecular networking is used to analyze unknown compounds by grouping compounds based on their fragmentation spectra similarity. Figure 2 represents the collective molecular network profile of the acetone and methanol extracts of MS-10 and MS-13. Distinct clusters of compounds were observed possibly representing either different classes or family of compounds which requires further exploration.



(a) (b)

Figure 2. Molecular networking of Antarctic mosses based on LC-MS/MS: (a) Clusters of networks representing compounds with similar fragmentation spectra; (b) Cluster highlighted in yellow indicating predicted compounds sharing similar structures.

3.3. COX-2 inhibition activity

COX-2 activity for each sample treatment was expressed as percentage value with respect to the 100% activity control (Figure 3). COX-2 activity was lowest for MS-13 acetone and MS-10 methanol extracts suggesting presence of COX-2 inhibitors. This underscores the potential of Antarctic moss samples, specifically MS-10 and MS-13, in the development of natural product-based anti-inflammatory treatments.

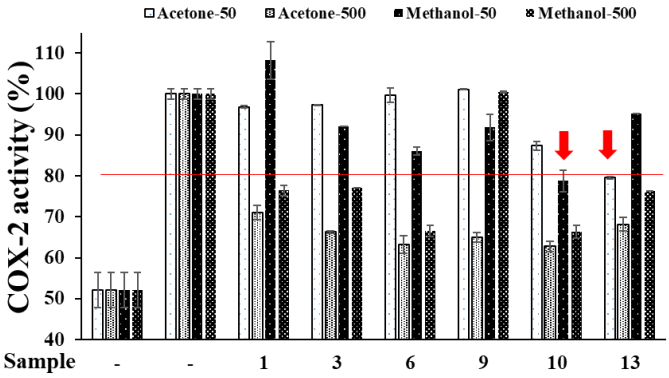


Figure 3. Inhibitory effect of acetone and methanol extracts of different moss samples on COX-2 enzyme activity.

4. Discussion

The increasing need for potent therapeutics in response to various health challenges underscores the significance of investigating underexplored sources such as Antarctic mosses. Utilizing compound databases constructed through extensive research, traditional medicine knowledge, and computational tools, it is now more efficient and relatively simpler to explore the chemical space of these resources, aiding in drug discovery and development [8, 31].

Metabolomics, specifically untargeted metabolomics, aims to characterize and categorize metabolites or compounds present in complex samples like crude extracts by comparing their mass spectral and/or NMR data with established reference databases. Through this approach, this study enabled us to explore the therapeutic potential of Antarctic mosses. Using solvents with differing polarity, acetone and methanol, compounds varying in chemical properties could be obtained as observed in the chromatogram suggesting that the Antarctic moss samples differed in their chemical compositions. Indeed, subsequent LC-MS/MS analysis and chemical compound annotation found that each sample extract contained compounds that were unique to the moss sample (data not shown). Additionally, compounds with known therapeutic potential including some FDA approved drugs were

identified within the Antarctic moss samples. However, the list only encompassed a fraction of the known compounds. A large portion of the extracts contained numerous unknown compounds, prompting the utilization of computational tools capable of clustering compounds based on similarities in their fragmentation patterns. This facilitated the creation of network structures representing known and unknown compounds, offering initial insights into the structural properties of the unknown compounds. Following experimental validation revealed that certain samples (MS-10 methanol extract and MS-13 acetone extract) exhibited COX-2 activity inhibition, indicating their potential therapeutic efficacy. Overall, the findings suggest that among the six Antarctic mosses, MS-10 and MS-13 contained bioactive compounds, specifically potent COX-2 inhibitors, implying their potential use in anti-inflammatory therapeutics.

The implications of these findings extend to addressing global health challenges and mitigating the imbalance between health risks and available therapeutics. The pharmaceutical industry faces significant hurdles in drug development, and exploring natural products from Antarctic mosses holds promise for discovering unique compounds that could contribute to the drug discovery process.

However, the study primarily serves as a preliminary screening method for bioactive compounds. While Antarctic mosses demonstrate potential for uncovering natural treatments for various diseases, more advanced techniques are necessary for identifying and discovering novel compounds. Future research efforts will involve isolating and establishing structure-activity relationships (SAR) of specific bioactive compounds to enhance our understanding of their mechanisms of action.

5. Conclusions

Traditional knowledge and advancements are the two sides of a coin. In the realm of medicinal research, integrating both aspects can unravel new avenues for therapeutic exploration. Through a comprehensive metabolomics approach (the knowledge and advancement), this study has shed light on the potential of Antarctic mosses (the unknown) as a source of bioactive compounds (the acknowledged) with promising therapeutic applications. By bridging traditional knowledge with modern analytical techniques, it became possible to uncover a rich reservoir of metabolites within these resilient plants, offering a glimpse into their therapeutic potential. Our findings underscore the importance of interdisciplinary approaches in drug discovery, where the convergence of traditional wisdom and scientific innovation can lead to novel therapeutic interventions. As we navigate the challenges of addressing evolving health concerns, exploring the untapped resources of our natural world, such as Antarctic mosses, holds great promise for the development of future pharmaceuticals. It is our hope that this study serves as a catalyst for further research, inspiring collaboration between traditional knowledge holders and scientific communities to unlock nature's pharmacopeia and improve global health outcomes.

Finally, it is important to remember that nature has interconnected various species by endowing mutual benefits, enabling them to address each other's challenges; thus, uncovering therapeutic significance and preservation of these vital ecosystems from extinction amid the changing environment ought to go hand in hand towards maintaining a sustainable environment.

Author Contributions: Conceptualization, K.L., R.Y. and T.-J.O.; methodology, K.L., R.Y.; formal analysis, K.L., R.Y. and H.Y.; investigation, K.L., R.Y. and H.Y.; resources, T.-J.O.; writing-original draft preparation, K.L., R.Y., H.Y. and T.-J.O.; writing-review and editing, K.L., R.Y., H.Y. and T.-J.O.; supervision, T.-J.O.; project administration, T.-J.O.; funding acquisition, T.-J.O. All authors have read and agreed to the published version of the manuscript.

Funding: This research was supported by the project titled "Development of potential antibiotic compounds using polar organism resources (20200610)", funded by the Ministry of Oceans and Fisheries, Korea. In addition, this work was supported by the MISP (Ministry of Science, ICT & Future Planning), Korea, under the National Program for Excellence in SW supervised by the IITP (Institute for Information & communications Technology Promotion) (Grant no. 2018-0-01865).

Data Availability Statement: Not applicable.

Conflicts of Interest: The authors declare no conflict of interest.

References

1. Traditional medicine has a long history of contributing to conventional medicine and continues to hold promise. https://www.who.int/news-room/feature-stories/detail/traditional-medicine-has-a-long-history-of-contributing-to-conventional-medicine-and-continues-to-hold-promise (accessed on 10 January 2024).

2. Petrovska, B. Historical review of medicinal plants' usage. *Pharmacogn Rev. 6*(11), 1–5 (2012). https://doi.org/10.4103/0973-7847.95849
3. Dias, D., Urban, S. and Roessner, U. A Historical Overview of Natural Products in Drug Discovery. *Metabolites 2*(2), 303–336 (2012). https://doi.org/10.3390/metabo2020303
4. Beilinson, H. TRADITIONAL MEDICINE: Using our Past to Advance Our Future. https://yaledistilled.sites.yale.edu/browse-issues/2019-issue/traditional-medicine-using-our-past-advance-our-future (accessed on 10 January 2024).
5. Domingo-Fernández, D. et al. Exploring the known chemical space of the plant kingdom: insights into taxonomic patterns, knowledge gaps, and bioactive regions. *J Cheminform 15*(1), (2023). doi: 10.1186/s13321-023-00778-w
6. Howes, M. et al. Molecules from nature: Reconciling biodiversity conservation and global healthcare imperatives for sustainable use of medicinal plants and fungi. *PLANTS, PEOPLE, PLANET 2*(5), 463–481 (2020). https://doi.org/10.1002/ppp3.10138
7. Gaudêncio, S. et al. Advanced Methods for Natural Products Discovery: Bioactivity Screening, Dereplication, Metabolomics Profiling, Genomic Sequencing, Databases and Informatic Tools, and Structure Elucidation. *Mar Drugs 21*(5), (2023). https://doi.org/10.3390/md21050308
8. Sadybekov, A. and Katritch, V. Computational approaches streamlining drug discovery. *Nature 616*(7958), 673–685 (2023). https://doi.org/10.1038/s41586-023-05905-z
9. Chen, Y., Garcia de Lomana, M., Friedrich, N. and Kirchmair, J. Characterization of the Chemical Space of Known and Readily Obtainable Natural Products. *J Chem Inf Model 58*(8), 1518–1532 (2018). https://doi.org/10.1021/acs.jcim.8b00302
10. Beckonert, O. et al. Metabolic profiling, metabolomic and metabonomic procedures for NMR spectroscopy of urine, plasma, serum and tissue extracts. *Nat Protoc 2*(11), 2692–2703 (2007). https://doi.org/10.1038/nprot.2007.376
11. Han, W., Ward, J., Kong, Y. and Li, X. Editorial: Targeted and untargeted metabolomics for the evaluation of plant metabolites in response to the environment. *Front Plant Sci. 14*, (2023). https://doi.org/10.3389/fpls.2023.1167513
12. Kontou, E. et al. UmetaFlow: an untargeted metabolomics workflow for high-throughput data processing and analysis. *J Cheminform. 15*(1), 52 (2023). https://doi.org/10.1186/s13321-023-00724-w
13. Demarque, D. P. et al. Mass spectrometry-based metabolomics approach in the isolation of bioactive natural products. *Sci Rep. 10*(1), 1051 (2020). https://doi.org/10.1038/s41598-020-58046-y
14. Duan, L. et al. Application of metabolomics in toxicity evaluation of traditional Chinese medicines Milen I. Georgiev. *Chinese Medicine (United Kingdom) 13*(1), 60 (2018). https://doi.org/10.1186/s13020-018-0218-5
15. Wang, D. et al. Metabolomics combined with network pharmacology exploration reveals the modulatory properties of Astragali Radix extract in the treatment of liver fibrosis. *Chinese Medicine (United Kingdom) 14*(1), 30 (2019). https://doi.org/10.1186/s13020-019-0251-z
16. Zuo, J. et al. Integrating Network Pharmacology and Metabolomics Study on Anti-rheumatic Mechanisms and Antagonistic Effects Against Methotrexate-Induced Toxicity of Qing-Luo-Yin. *Front Pharmacol. 9*, (2018). https://doi.org/10.3389/fphar.2018.01472
17. Gutt, J. et al. Antarctic ecosystems in transition – life between stresses and opportunities. *Biological Reviews 96*(3), 798–821 (2021). https://doi.org/10.1038/nprot.2007.376
18. Turini, M. and DuBois, R. Cyclooxygenase-2: A Therapeutic Target. *Annu Rev Med. 53*(1), 35–57 (2002). https://doi.org/10.1146/annurev.med.53.082901.103952
19. Bashir, K. et al. Tandem high resolution mass spectrometry based phytochemical composition of Sauromatum guttatum tubers and its enzyme inhibitory potential with molecular docking. *J Chromatogr A 1672*, 463055 (2022). https://doi.org/10.1016/j.chroma.2022.463055
20. Morehouse, N. et al. Annotation of natural product compound families using molecular networking topology and structural similarity fingerprinting. *Nat Commun. 14*(1), 308 (2023). https://doi.org/10.1038/s41467-022-35734-z
21. Ponomareva, L., Timoshkina, N., Sarantseva, L. and Bulanov, A. Clinical experience in using cereton in ischemic stroke and dyscirculatory encephalopathy. *Neurology, neuropsychiatry, Psychosomatics 2*(2), 62-64 (2010). https://doi.org/10.14412/2074-2711-2010-87
22. Catanesi, M. et al. Neuroprotective potential of choline alfoscerate against β-amyloid injury: Involvement of neurotrophic signals. *Cell Biol Int. 44*(8), 1734–1744 (2020). https://doi.org/10.1002/cbin.11369
23. Johansson, B. A review of the pharmacokinetics and pharmacodynamics of disulfiram and its metabolites. *Acta Psychiatr Scand. 86*(S369), 15–26 (1992). https://doi.org/10.1111/j.1600-0447.1992.tb03310.x
24. Muthukumaran, P., Thiyagarajan, G., Arun Babu, R. and Lakshmi, B. Raffinose from Costus speciosus attenuates lipid synthesis through modulation of PPARs/SREBP1c and improves insulin sensitivity through PI3K/AKT. *Chem Biol Interact. 284*, 80–89 (2018). https://doi.org/10.1016/j.cbi.2018.02.011
25. Meglutol. https://go.drugbank.com/drugs/DB04377 (accessed on 10 January 2024).
26. El-Demerdash, E. Anti-inflammatory and antifibrotic effects of methyl palmitate. *Toxicol Appl Pharmacol. 254*(3), 238–244 (2011). https://doi.org/10.1016/j.taap.2011.04.016
27. Mendelson, W. The Hypnotic Actions of the Fatty Acid Amide, Oleamide. *Neuropsychopharmacology 25*(5), 36–39 (2001). https://doi.org/10.1016/S0893-133X(01)00341-4

28. Nam, H., Na, E., Lee, E., Kwon, Y. and Kim, H. Antiepileptic and Neuroprotective Effects of Oleamide in Rat Striatum on Kainate-Induced Behavioral Seizure and Excitotoxic Damage via Calpain Inhibition. *Front Pharmacol. 8*, 817 (2017). https://doi.org/10.3389/fphar.2017.00817
29. Zhang, X. et al. Advances in Stigmasterol on its anti-tumor effect and mechanism of action. *Front Oncol. 12*, 1101289 (2022). https://doi.org/10.3389/fonc.2022.1101289
30. Wang, J. et al. Anti-diabetic activity of stigmasterol from soybean oil by targeting the GLUT4 glucose transporter. *Food Nutr Res. 61*, (2017). https://doi.org/10.1080/16546628.2017.1364117
31. Han, R., Yoon, H., Kim, G., Lee, H. and Lee, Y. Revolutionizing Medicinal Chemistry: The Application of Artificial Intelligence (AI) in Early Drug Discovery. *Pharmaceuticals 16*(9), 1259 (2023). https://doi.org/10.3390/ph16091259