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Pan-Himalaya ethnomedicine safety: Lithospermeae (Boraginaceae) herbal remedies containing toxic pyrrolizidine alkaloids

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ABSTRACT

Aim/Background: Boraginaceae is famous for the production of pyrrolizidine alkaloids (PAs) and some of these PAs are carcinogenic and also cause liver failure. Therefore, the aim of the present study was to identify the presence or absence of hepatotoxic pyrrolizidine alkaloids in the tribe Lithospermeae (Boraginaceae). If any are found, it may indicate excluding members of this tribe from the herbal formulation or from use on patients with liver problems.

Materials and Methods: Plant samples of *Onosma hispida* Wall. ex G. Don, *Onosma paniculatum* Bureau & Franch., *Onosma hookeri* var. *longiflorum* (Duthie) A.V. Duthie ex Stapf, and *Maharanga emodi* (Wall.) A. DC. from Boraginaceae—Tribe Lithospermeae were collected from various regions of Pan Himalaya and brought to Beijing Normal University for further experimentation. We used acetonitrile–water gradient with 0.1% formic acid as the mobile phase and Zorbax SB-Aq column to analyze samples. Furthermore, we also searched the literature to find the ethnomedicinal importance of these plants.

Results: The results showed that these plants are used orally for the treatment of various human ailments, and, therefore, we further investigated these plants for toxic PAs. High-performance liquid chromatography results showed that leaves of these plants were PA positive, and out of four PA standards, three: Heliotrine (2), Lycopsamine (3), and Echimidine (4) were detected.

Conclusions: In this study, we present a new report about the presence of toxic PAs in the leaves of *O. hispida*, *O. paniculatum*, *O. hookeri* var. *longiflorum,* and *M. emodi* from the Pan-Himalaya region. These plants are used in traditional medicine mostly in Pakistan, Nepal, and China, and the presence of hepatotoxic PAs limits the use for medicinal purposes.

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Introduction

The knowledge of medicinal plants has been accumulated in the course of many centuries based on different medicinal systems, such as Ayurveda, Unani, and Siddha. Often, people of developing countries rely more on traditional medicine, possibly due to low access to modern health services [1]. These ethnomedicinal practices are transmitted from generation-to-generation and still practiced in various communities because of very low expense and good pharmacological results. These valuable medicinal plants contain rich bioactive compounds which have various pharmacological activities [2]. Indigenous people around the world depend on plants for their basic healthcare and economic values. These benefits are based on the experience of older native people, need, and observation [3]. Natural resources play a vital role to provide us food, fuel, shelter, clothing, and medications as well as different necessities of sustainable life to the humans [4,5]. Medicinal plants have served mankind by providing local remedies to

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treat ailments. Because of this, people have investigated medicinal properties throughout history [6].

In many developing countries, the safety of herbal medicine is also a major concern [7]. In Western countries, many alternative medicine practitioners claimed that traditional medicines show only benefits and they have no side effects. Therefore, herbal medicines and products may be ingested without taking the necessary precautions and, indeed, several fatal intoxication cases have been reported—the cause being the use of herbal remedies [8]. Unfortunately, the critical and testing role of pharmacovigilance in local ethnomedicinal markets is often lacking to non-existent [9]. These dangers can be magnified with issues of product quality, processing methods, miss identification of taxa, and knowledge of potential physiological effects [10]. Several cases of severe accidental poisoning by medicinal plants used as part of traditional treatment include neurological signs and sometime multi-organ failure [11]. Other poisonous cases due to herbal remedies have also been reported recently [12,13].

Pyrrolizidine alkaloids (PAs) are one of the common sources of herbal remedies to produce toxicity [14]. Pyrrolizidine alkaloids are secondary plant metabolites that are mostly found in the botanical families of Asteraceae, Boraginaceae, and Fabaceae and they form a powerful defense mechanism against herbivores [15,16]. Among 6,000 angiosperm species, approximately 600 have naturally occurring PAs [17]. It is a large group of toxins naturally synthesized by various plant species as secondary metabolites. Several toxic PAs enter into the food chain presenting hazards to humans and animals [18]. In developing and industrialized countries, the use of herbal medicine has become increasingly more common and so PA poisoning is one of the main problems reported within the last 25 years. Those PAs, which possess a 1, 2-double bond in their base moiety, are hepatotoxic, carcinogenic, teratogenic, genotoxic, and sometimes pneumotoxic [19]. The liver of humans can be damaged by acute poisoning with PAs, whereas a sub-acute dose may lead to pulmonary arterial hypertension and liver cirrhosis [20]. The hepatotoxic PAs, particularly 1, 2-unsaturated PAs, are undesirable in herbal products and other foods due to their acute and chronic liver-damaging effects [21]. Oral ingestion of PAs-containing herbal remedy or teas is the main cause of hepatic sinusoidal obstruction syndrome (HSOS). Serious HSOS leads to liver and multi-organ failure, so liver transplantation can be needed and even can result in death. There is still no effective strategy for HSOS treatment in the clinic [22]. PA metabolism occurs mainly in the liver, which is also the main target organ of toxicity [23]. Around the world, thousands of clinical cases of HSOS due to PAs-poisoning have been documented since 1920 [24,25]. In the last few years, PAs, especially in herbal medicinal products became a widely discussed issue and it is the reason that the European Medicinal Agency gives precautions to control PAs contaminating herbal and other food products [26].

Besides many other herbal drugs, *Onosma hispida*, *O. paniculatum*, *O. hookeri* var. *longiflorum*, and *Maharanga emodi* (Boraginaceae—Tribe Lithospermeae Dumort.) are also one of the main potential sources of PA toxicity. The genus *Onosma* L. is a species-rich genus which includes about 150 species all around the world [27]. *Onosma hispida* Wall. ex G. Don is a perennial herb up to 70 cm tall with a prominent taproot. The plant is distributed in Northern Pakistan Gilgit, Chitral, Swat, and Hazara [28]. *Onosma paniculatum* Bureau & Franch. is biennial, or rarely perennial herb with the single stem up to 40–80 cm tall [29]. The genus *Maharanga* is herbaceous, perennial, or biennial with nine species that distribute from middle Himalaya to the southern part of China [30]. The present study is carried out with the aim to determine the presence or absence of hepatotoxic pyrrolizidine alkaloids in *O. hispida*, *O. hookeri* var. *longiflorum*, *O. paniculatum*, and *M. emodi*. If these alkaloids are found, it would indicate exclusion from the herbal formulation.

Materials and Methods

Literature review of ethnomedicinal uses of selected plants

We collected data and surveyed relevant literature regarding the herbal remedies *O. hispida*, *O. paniculatum, O. hookeri* var. *longiflorum*, and *M. emodi* from Pakistan, China, and Nepal. For this, we queried the scientific databases of Web of Science and Google Scholar for the keywords in the search term "ethnomedicinal uses" or "medicinal uses" of *O. hispida*; "Boraginaceae ethnobotany," "medicinal uses of *M. emodi*," "medicinal uses of *O. paniculatum*," and "medicinal uses of *O. hookeri*."

Plant sample collecting sites—The Pan-Himalayan regions

The Pan-Himalayas (the Himalayas and adjacent regions) forms a natural geographic unit from the

Wakhan Corridor and the north-eastern Hindu Kush eastwards to the Hengduan Mountains by way of the Karakorum and the Himalayas. This region covers the north-eastern corner of Afghanistan, Nepal, northern India, northern Pakistan, northern Myanmar, southwest China, and Bhutan [31].

The northern parts of Pan-Himalaya of Pakistan include Azad Kashmir, Chitral, Swat, Dir, Hazara Division, and Gilget-Biltistan. We collected the plant samples of *O. hispida* Wall. ex G. Don from Bomboret, Chitral in July 2017. Bomboret Valley is located in the south-west of Chitral town district Chitral which lies in north-western Pakistan, and south of the Afghan Wakhan Corridor [32].

Similarly in southwest China, part of Pan-Himalaya includes SE Gansu, SE Qinghai, NW Yunnan, W Sichuan, and S Tibet [31]. The *M. emodi* was collected by Lai Wei and Jia-Chen Hao from Geelong county-Tibet, and *O. paniculatum* was collected from Zhongdian county Yunnan province by Jian-Fei Ye et al. While *O. hookeri* var. *longiflorum* was collected by Yi He and Dan-Hui Liu from Ngamring County, Tibet (Supplementary Material).

Plant collection, identification, and deposition in herbarium

The plant sample of *O. hispida* collected from northern parts of Pan-Himalaya of Pakistan (Chitral, Gilgit-Baltistan, Swat, Abbottabad, Azad Kashmir, and Dir). While, *M. emodi, O. paniculatum*, and *O. hookeri* var. *longiflorum* from southwest China, part of Pan-Himalaya along with other Lithospermeae-Boraginaceae members were brought to Beijing Normal University in Beijing, China. The plants were identified, voucher specimen numbers were assigned, and deposited at Herbarium of Beijing Normal University as a ready reference for future studies. The collector number, collector name, voucher specimen number, altitude, and collecting sites are represented in Table 1 (Supplementary Material).

Experimental

Chemicals, reagents, and standards

Methanol and acetonitrile were of high-performance liquid chromatography (HPLC) grade and purchased from Dikma Technologies Inc. (Lake Forest, CA). Formic acid was the product of Aladdin Industrial Corporation (Shanghai, China). Pure water used throughout the experiment was prepared from a Milli-Q water purification system (Millipore Corporation, Billerica, MA). Reference standards of PAs; echimidine, heliotrine, lycopsamine, and europine were purchased from ChemFaces (Hubei, China) and had a purity > 98%.

Botanical samples preparation

The ethnomedicinal plant samples for HPLC were prepared according to well-known published methods [33,34]. Briefly, dried leaves (500 mg) of mature flowering *O. hispida, O. paniculatum*, *O. hookeri* var. *longiflorum*, and *M. emodi* were accurately weighed and crushed in a mortar and pestle with liquid nitrogen. The sample was sonicated for 35 minutes with 3 ml of methanol immediately followed by centrifugation for 15 minutes at 4,000 *g*. After centrifugation, the supernatant was transferred to another clean tube. The above procedure was repeated thrice and the respective supernatants combined into the same tube. The final volume was adjusted to 10 ml with methanol and mixed thoroughly. Prior to injection, 300 µl of the sample was passed through a 13 mm × 0.45 µm FitMax Syringe filter membrane Nylon (Dikma, USA).

Standard preparation

The stock solutions of the following standards were prepared separately at concentrations of 1.0 mg mL^{-1}: lycopsamine, heliotrine, europine, and echimidine [33–35].

Table 1. Plant samples collected from research sites in Pan-Himalaya regions.

Voucher No.	Scientific name	Collecting site	Altitude (m)
BNU0033440	*O. hispida* Wall. ex G. Don	Bomborait, Chitral, Pakistan	2,151
BNU0033442	*O.paniculatum* Bureau. & Franch	Zhongdian county; Yunnan; China	
BNU0033441	*O. hookeri* var. longiflorum (Duthie)	Ngamring county, Tibet.	4,693
BNU0033439	*M. emodi* (Wall.) A. DC.	Geelong county; Tibet; China	3,977

Instrumentation and conditions

The liquid chromatographic system was the Waters Alliance 2695 LC System with 2487 Dual Wavelength UV Detector (Milford, MA) comprised of the following modular components: 4 channel degasser, built-in quaternary pump, auto-injector, and auto-sampler with 120 2 ml vials. We used a Zorbax SB-Aq (4.6 × 250 mm, 5 µm particles) column (Agilent, USA) for separation of the PAs from *O. hispida*, *O. paniculatum*, *O. hookeri* var. *longiflorum*, and *M. emodi*.

A gradient LC method was developed for the PAs analysis in the leaves of *O. hispida, O. paniculatum*, *O. hookeri* var. *longiflorum,* and *M. emodi*. The mobile phase compositions of the HPLC system were: (A) water with 0.1% formic acid and (B) acetonitrile with 0.1% formic acid at a flow rate of 0.5 ml/minute with the gradient elution system of A and B (87:13; 50:50; 13:87 in 53 minutes). Before moving to the next sample, the system was washed by methanol for 15 minutes. The temperature of the sample tray and column was at room temperature, the injection volume was 10 µl, and the detection wavelength was set at 280 nm.

Results

Ethnomedicinal uses of the Onosma species and M. emodi

In our previous study, the decoction of the aerial parts was used to treat hypertension [36]. Recently, Sher et al. [32] reported a new traditional remedy for *O. hispida.* In this remedy, powder of the whole plant is taken with a glass of milk for quick recovery after delivery of a baby. Similarly, *M. emodi*, known as Marangi in Nepali, is a well-known herb used in Nepalese traditional medicine [37–39]. In traditional Chinese medicine, *Zicao* is a traditional remedy for the treatment of cancer, and *O. paniculatum* is one of the main herbs in the preparation of *Zicao* [40]. Other ethnomedicinal uses of these plants are shown in Table 2.

Separation and determination of PAs in Onosma species and M. emodi

Four ethnomedicinal plant samples of *O. hispida*, *O. paniculatum*, *O. hookeri* var. *longiflorum,* and *M. emodi*, commonly used in traditional medicine in Pakistan, Nepal, and China were phytochemically

Table 2. Ethnomedicinal uses of Pan-Himalayan species from Chinese and Pakistani regions.

Scientific name	Part used	Medicinal Uses	Literature
O. hispida Wall. ex G.Don	Whole plant	Quick recovery after delivery	Sher et al. [32]
	Aerial Part	Hypertension	Ahmad et al. [36]
	Whole plant	Jaundice and liver diseases	Sher et al. [41]
		Medicinal use (not mention specific disease)	Ahmad et al. [42]
	Root extract	Pneumonia and typhoid fever	Khan and Khatoon [43]
O. paniculatum Bureau & Franch.	Plant extract	Cancer	Rinner et al. [40]
	Root	Nettle rash, acute and chronic hepatitis	Ning and Cao [44]
		Acute chronic hepatitis, pulmonary tuberculosis, gynecologic inflammation infant dermatitis	Jiangsu New Medical College [45]
O. hookeri var. *longiflorum*	Root	Pulmonary tuberculosis	Gu [46]
(Duthie) A.V. Duthie ex stapf.		Pneumonia	Luo [47]
		Anti-inflammation, contraception, antineoplastic	Tibet Health Bureau [48]
M. emodi (Wall.) A. DC.	Whole plant	Hypertension, fever, and blood purification	Pandey [37]
	Root	Anti-viral activity	Rajbhandari et al. [49]
		Hair tonic	Bhattarai [38]

investigated qualitatively for PAs. These plants were collected from different sites of Pan-Himalaya.

In our study, all the samples were processed, using the HPLC conditions optimized for the plant samples and reference standards stated above. HPLC methods are non-destructive and have the advantage of allowing the determination of free bases and N-oxides in a single analytical run without prior reduction of the oxides, thus both the preparation and analysis stages are much reduced [50]. Four reference standards of PAs, namely, europine (1), heliotrine (2), lycopsamine (3), and echimidine (4) were used to identify the possible PAs in leaves of the investigated plant species and their chemical structures shown in the Figure 1. In our previous study of PAs from *Arnebia benthamii* tribe *Lithospermeae* (Boraginaceae), we used the same HPLC machines conditions for these new samples [14]. Using the above stated HPLC conditions, the four standards of europine, heliotrine, lycopsamine, and echimidine were separated with retention times of 10.21, 10.77, 37.16, and 46.64 minutes, respectively**.** After identification of the retention time of standards, 10 µl of each plant samples was injected and the retention times were compared to those of reference standards.

The qualitative analyses of four PA compounds in *O. hookeri* var. *longiflorum* and *O. hispida* show positive for three PAs, i.e., heliotrine, lycopsamine, and echimidine. Similarly, *O. paniculatum* were positively detected for the two PAs lycopsamine and echimidine. While two PAs lycopsamine and echimidine were found in *M. emodi*. Figure 2 represents the chromatogram of the mixture and of the four samples.

Discussion

In the present study, we have investigated the pyrrolizidine alkaloid profile of the leaves of *O. hispida*, *O. paniculatum*, *O. hookeri* var. *longiflorum,* and *M. emodi* from Pan-Himalaya and, for the first time, reported this in the literature. However, PAs have been previously reported from some other *Onosma* spp. such as 3′-O-acetylechinatine N-oxide from O. *kaheirei* Teppner [51], intermedine and lycopsamine in *O. alborosea,* 7-acetylintermedine, lycopsamine, and intermedine in *O. arenaria* subsp. *pennina* [52], 7-Acetyllycopsamine, 5, uplandicine, 7-acetylretronecine etc. in *O. arenaria* [53], viridinatine and onosmerectine in *O. erecta* [54], heliotridine in *O. hetrophyllum* [55], eptanthine and echihumiline in *O. leptantha* [56], and lycopsamine, leptanthine, echimidine, heliospathuline, and 7-viridiflorylretronecine in *O. stellulatum* [57,58]. To the best of authors' knowledge, genus *Maharanga* is investigated for the first time to determine PAs. In the past, Roeder and Wiedenfeld [39] alluded to this by proposing that the genus *Maharanga* is similar to *Onosma* and that plants of this genus have toxic alkaloids.

Many PA-containing plants and individual PA compounds have been tested in animal models and

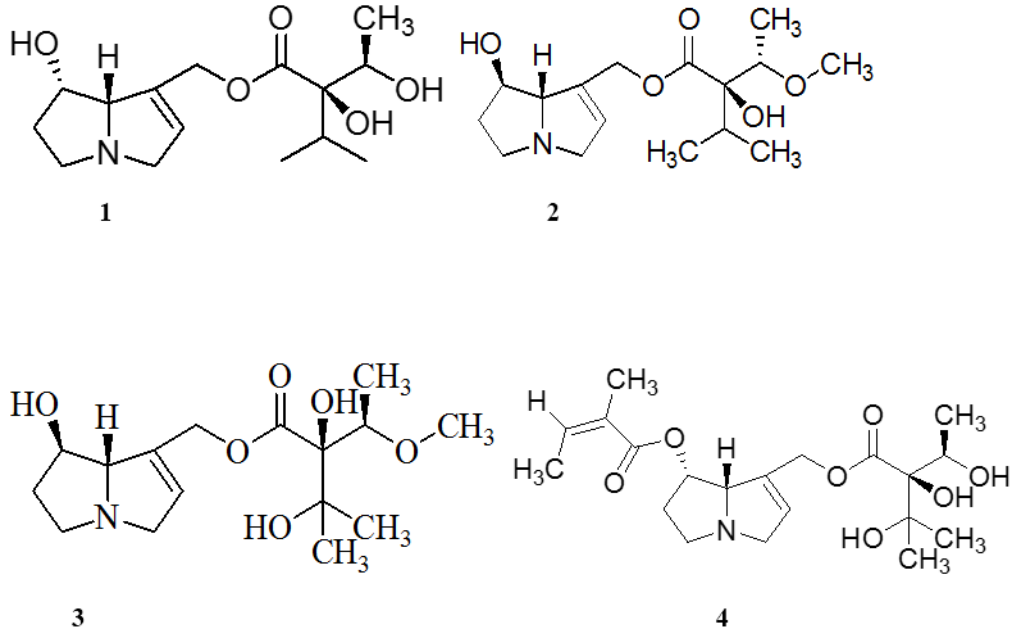

Figure 1. Structure of authentic standards of pyrrolizidine alkaloids.

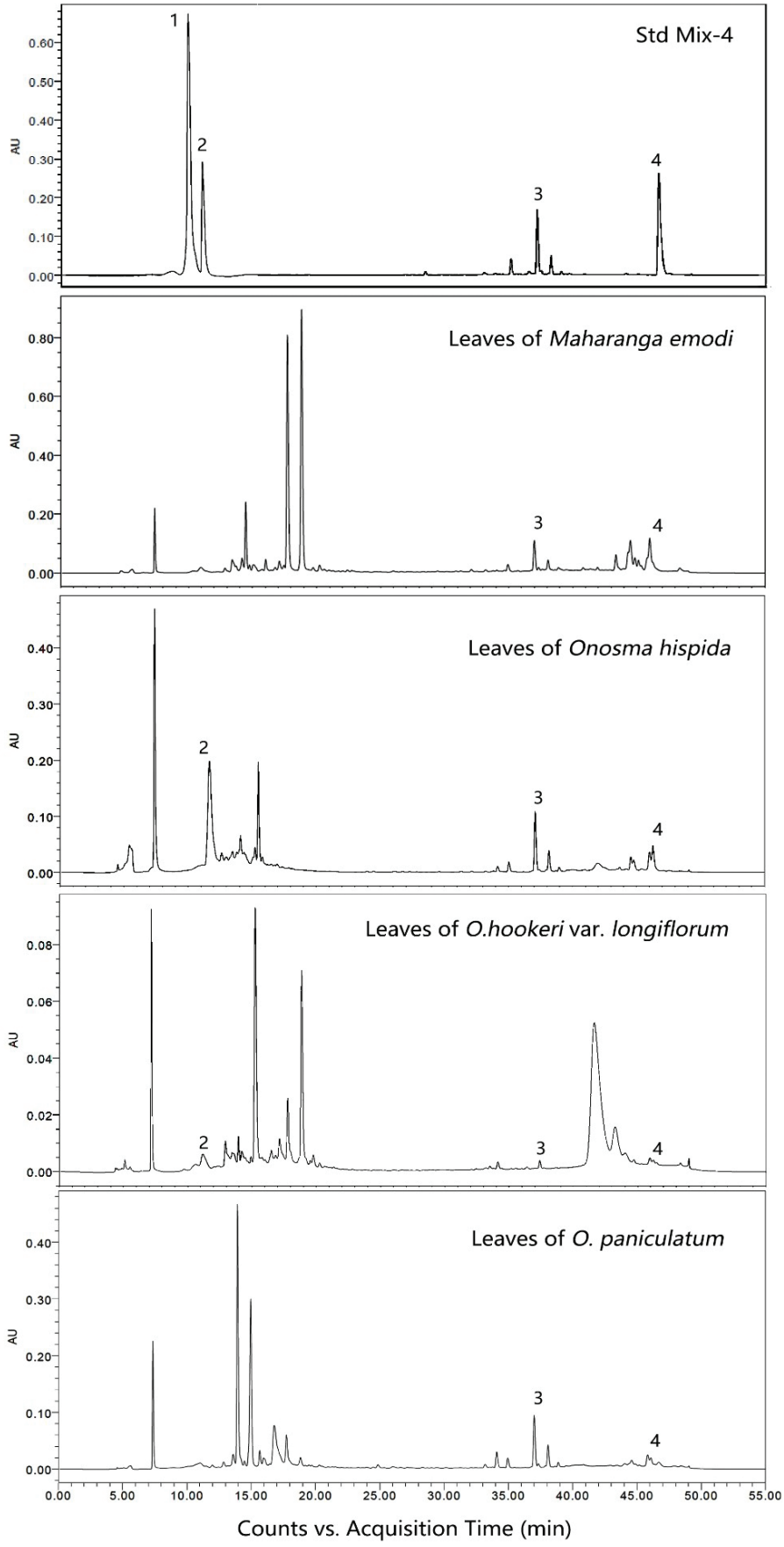

Figure 2. Base-peak chromatograms of standard mix and leaves of *M. emodi*, *O. hispida*, *O. hookeri* var. *longiflorium*, and *O. paniculatum.*

shown to be carcinogenic in various tissues. The liver is the main carcinogenic target [17]. Lycopsamine is one of the heptotoxic PAs and it damages the liver [59]. Additionally, heliotrine is classified as a heliotridine-type PA; these have been shown to induce mutagenesis and liver tumors, be carcinogenic to the liver, and also damage chromosomes [8,17,60]. Echimidine is a hepatotoxic pyrrolizidine alkaloid [61]. Unfortunately, in our study, these two toxic PAs are found in all four of the investigated species.

Pyrrolizidine alkaloids are typical metabolites of the family Boraginaceae present usually in the form of their N-oxides that are hydrophilic because of polar compounds [62]. These N-oxides cannot be directly converted to the hydroxy-PAs, but whenever humans or livestock intake PA containing plant parts, they are reduced by the gut enzymes or the liver microsomes and NADH or NADPH to the free bases and metabolic activation forms hepatotoxic pyrroles. Therefore, they show equal toxicity to that of the free bases and cause acute and chronic effects in man and livestock [63–65]. Unfortunately, in our study, all four investigated species *O. hispida*, *O. paniculatum*, *O. hookeri* var. *longiflorum,* and *M. emodi* were reported in the literature to be used orally for the treatment of various human ailments**.** In our previous study, the decoction of the leaves of *O. hispida* was used by the traditional people in Dir, Pakistan, for the treatment of hypertension [36]. Similarly, for another three species, oral ingestion is also reported as shown in Table 2.

There are a large numbers of reports on PA poisoning and intoxication in humans. Previously, it has been well-established that PA containing plants and contaminated food affect both developing and developed countries. In the 1920s in South Africa, liver disease was widespread and it was caused by the consumption of bread contaminated with PAs from seeds from *Senecio* species [66]. Similarly in 1968, in the same country, 15 children had the veno-occlusive disease (VOD) by using bush-teas with *Crotalaria* spp., out of 15, 10 children died [67]. In 1954, 23 adults had VOD in Jamaica be caused by bush-teas with *Crotalaria fulva* [68]. In 1970 in Iraq, 9 children have VOD because of food contaminated by a *Senecio* spp. [69]. In Afghanistan in 1970–1972, contaminated wheat with *Heliotropium popovii*, ssp. *gillianum* made approximately 7,200 people to suffer from VOD [70]. Similarly, 3,906 people suffered abdominal pain, hepatomegaly, ascites, alteration of consciousness, and were hospitalized in Tajikistan because of *Heliotropium lasiocarpum* contamination of grain in 1992 [71]. In India, in 1974–1977, six people had VOD because of contaminated food with *Heliotropium eichwaldii* [72]. Intoxication in humans due to PA containing plants and contaminated food were also established in well-developed countries like the USA, the UK, Switzerland, China, Argentina, and Austria [73–78].

Based on reports about diseases and intoxications to human as well as livestock from PAs around the globe, the European Medicines Agency (EMA) has implemented a limit of intake of PAs from herbal medicinal products (i.e., 1 μg/day) as a transitional measure for 3 years, after which the threshold will be set to 0.007 μg of 1, 2-unsaturated PA/kg body weight [79,80]. In the European Union, the so-called "zero-tolerance principle" can be applied; this principle is used in cases where no safe or tolerable level can be determined based on available, valid scientific data, or if insufficient toxicological data are available. Due to their genotoxic and carcinogenic potential, this principle can be applied for PA in food and fodder [81]. In our study, we have limited information about safe or tolerable levels of the studied species containing PAs. We also have insufficient data about toxicity and quantitative analysis of *O. hispida, O. paniculatum, O. hookeri* var. *longiflorum,* and *M. emodi*. So as for the suggested principle by Bundesinstitut für Risikobewertung [81], we also suggest the ''zero-tolerance principle'' be applied for the investigated species before the quantitative analysis of PAs.

Conclusion

This study identified, for the first time, PAs in the leaves of *O. hispida*, *O. paniculatum*, *O. hookeri* var. *longiflorum*, and *M. emodi* from Pan-Himalaya region. The selected plants were found to be positive of hepatotoxic Pas, such as heliotrine, lycopsamine, and echimidine. Our results show that besides their ethnomedicinal value, the species is also a source of hepatoxic PAs. Because of insufficient data in the literature about the toxicity of these plants, quantity of PAs, and non-availability of tolerable or safe level, we suggest that the "zero-tolerance principle" should be applied. Furthermore, we recommend that these plants be excluded from local markets and their herbal formulations should not be sold before PA safe levels or tolerable levels are determined.

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Conflict of interest

The authors declare that they have no competing interest.

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e-Supplementary Material

For the last few years, our lab has been working on the taxonomic revision and importance of the family Boraginaceae for the "*Flora of Pan-Himalaya*". In July 2016–2018, a plant collection trip was arranged to collect members of Boraginaceae in the Pakistani Pan-Himalayan regions. In addition, samples from southwest China, part of Pan-Himalaya, from last 5 years for the project of the "*Flora of Pan-Himalaya*" were used (Fig. S1). July to August were selected because it is the peak flowering and fruiting season for Boraginaceae members. In the field, whole plants were collected and pressed for herbarium specimens, and in a paper bag, fresh plant material was collected in silica gel for molecular and phytochemical analysis. Dr. Latif Ahmad is of Pakistani nationality so we decided to have him cover the Pakistani Pan-Himalaya region in Northern Pakistan, while Chinese students were assigned to investigate Chinese Himalayan regions.

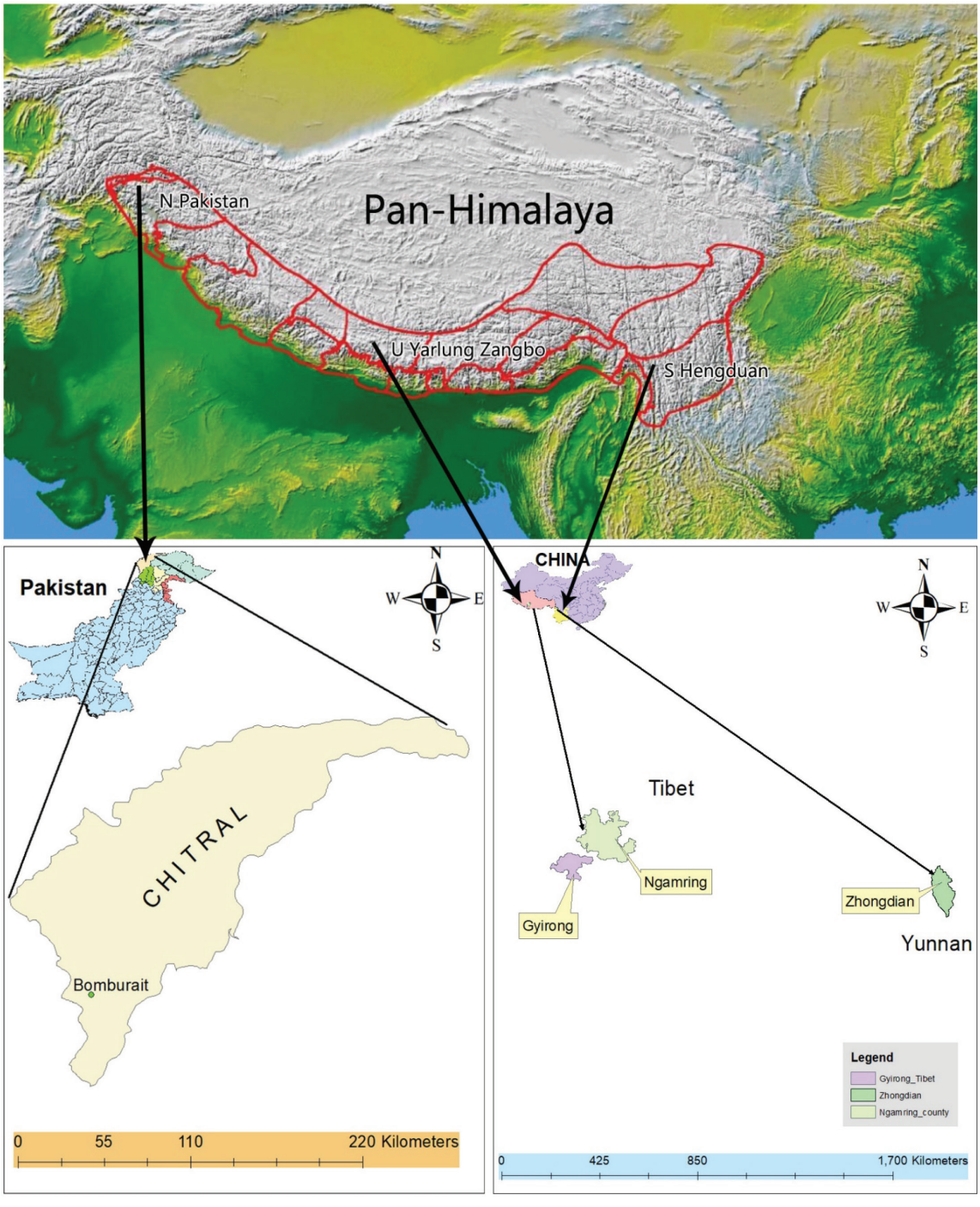

Figure S1. Map of the plants collected sites (Pan-Himalaya part taken from source: www.flph.org).

From literature searches, we know that Boraginaceae is famous for Pyrrolizidine alkaloids (PAs) with some of them being carcinogenic and also causing liver failure. Our interest lies in those species which are used in herbal remedies. For this, we also asked during interviews, about their ethnomedicinal value in addition to that presented in the studied literature. The tribe Lithospermeae of Boraginaceae has many ethnomedicine which are used for a plethora of herbal remedies.

We collected 26 Lithospermeae members from Chinese and Pakistani Pan-Himalaya regions and brought them to Beijing Normal University for identification and experimental work. We targeted those species which have ethnomedicinal value and are not being studied for toxic pyrrolizidine alkaloids. Among 26 species of Lithospermeae, we select four ethnomedicines *O. hispida* Wall. ex G. Don, *O. paniculatum* Bureau & Franch., *O. hookeri* var. *longiflorum* (Duthie) A.V. Duthie ex Stapf, and *M. emodi* (Wall.). *Onsoma hispida* is a well-known herbal remedy in Northern Pakistan, while Maharanga has medicinal value in Nepal, *O. paniculatum* and *O. hookeri* var. *longiflorum* are widely used in traditional Chinese medicine (Fig. S2).

After reviewing the literature on the above species that we selected, *O. paniculatum, O. hookeri* var. *longiflorum, M. emodi*, *O. hispida,* for investigation of toxic PAs, we organized them into a table for clear reference. Members of Lithospermeae are presented in Table S1.

Figure S2. (a)–(b) *Onosma hookeri* var. *longiflorum* (Duthie) A.V. Duthie ex Stapf, **(c)–(d)** *Maharanga emodi* (Wall.) A. DC., **(e)–(f)** *Onosma hispida* Wall. ex G. Don, and **(g)–(h)** *Onosma paniculatum* Bureau & Franch.

Table S1. List of Lithospermeae members collected from different Pan-Himalaya regions.

Collector No.	Plant name	Collection sites	Altitude (m)
LA-95BNU	*O. hispida* Wall. ex G. Don	Bomborait, Chitral, Pakistan	2,151
CH-03 BNU	*Onosma dichroantha* Boiss.	Chitral Goal, Chitral, Pakistan	1,642
LA-29BNU	*Onosma hypoleucum* I.M. Johnst.	Toli pir, Azad Kashmir, Pakistan	2,446
LA-87BNU	*Onosma griffithii* Vatke	Astore, Gilgit-Biltistan, Pakistan	3,384
LA-64BNU	*Onosma thomsonii* Clarke	Malam Jaba, Swat, KPK, Pakistan	2,521
CH-06 BNU	*Onosma chitralicum* I.M. Johnst.	Chitral, Khyber Puktunkhwa, Pakistan	1,620
BNU2017XZ052	*O. hookeri* C.B. Clarke	Angren county, Tibet, China	4,693
20110804040	*O. paniculatum* Bureau & Franch.	Zhongdian county; Yunnan; China	Unknown
BNU2017XJ383	*Onosma apiculatum* Riedl	Zhaosu county, Xinjiang , China	2,178
BNU2017XJ153	*Onosma gmelinii* Ledeb.	Altai City, Xinjiang , China	815
XMLY12019	*Onosma maaikangense* W.T. Wang	Maerkang county, Sichuan ; China	2,650
15107	*Onosma confertum* W.W. Sm.	Daocheng county; Sichuan ; China	3,579
XMLY12033	*Onosma liui* Kamelin & T.N. Popova	Rangtang county; Sichuan ; China	2,938
HY2017015	*Onosma sinicum* Diels	Wenxian county, Gansu, China	860
15576	*Onosma multiramosum* Hand.-Mazz.	Zuogong county, Tibet, China	4,000
15583	*Onosma adenopus* I.M. Johnst.	Mangkang county, Tibet, China	3,508
BNU2017XZ326	*Onosma waddellii* Duthie	Qushui county, Tibet, China	3,720
LA-49BNU	*A. benthamii* (Wall. ex) G. Don . I.M. Johnst.	Taiobat, Nellum, Azad Kashmir, Pakistan	2,415
BNU2017XJ125	*Arnebia guttata* Bunge	Fuxun county, Xinjiang , China	1,140
LA-82BNU	*Arnebia euchroma* (Royle) I.M. Johnst.	Below Deosai Top, Skardu, Gilgit-Biltistan, Pakistan	4,037
LA-67BNU	*Arnebia hispidissima* (Lehm.) A. DC.	Tooq, Mustooj, Chitral, Pakistan	2,321
15361	*M. emodi* (Wall.) A. DC.	Geelong county; Tibet; China	3,977
15431	*Maharanga bicolor* (Wall. ex G. Don) A. DC.	Geelong county; Tibet; China	3,500
LA-51BNU	*Lithospermum officinale* L.	Taiobat, Azad Kashmir, Pakistan	2,315
LA-53BNU	*Lithospermum arvense* L.	Swat, Khyber Puktunkhwa, Pakistan	1,241
LA-85BNU	*Lithospermum tenuiflorum* L.f.	Swat, Khyber Puktunkhwa, Pakistan	1,175

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SHORT COMMUNICATION

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Mitigating and anti-apoptotic effect of turmeric on semicarbazide-induced damage in testicular tissue of juvenile male rats

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ABSTRACT

Background: Semicarbazide (SE) is a known food additive that can be found both in nature and in manufactured glass containers. Unfortunately, it has deleterious effects on both animals and human.

Methods: Male juvenile rats were divided into four experimental groups: control group, TU group, SE group, and SE + TU group. In addition to the histological examination, blood total triiodothyronine (T3), total thyroxine (T4), testosterone levels, histological picture of testis, as well as the expression of caspase-3 and Bax were investigated.

Results: The administration of SE caused many damages, including weight loss, decrease in testes weight, and histopathological damages in the testicular tissue. In contrast, the combined administration of SE + TU restored the T3, T4, and testosterone levels as well as improved the histological testicular profile.

Conclusion: Therefore, it is concluded that TU was able to mitigate the deleterious effects of SE.

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KEYWORDS

Bax; caspase-3; juvenile rats; semicarbazide; testis; turmeric

Introduction

Semicarbazide (SE) is a hydrazine derivative by-product of azodicarbonamide, a known food additive due to its dough-improving properties [1]. It is found naturally in eggs, algae, and shrimps and can be formed from arginine and creatine [2]. SE is formed in processed food due to the treatment with hypochlorite and food additives [2,3]. It can also be present in glass containers, such as jars and bottles, which are foamed using the azodicarbonamide as blowing agent [4].

It has been experimentally shown that SE acts as an inhibitor of many enzymes in vertebrates, reducing their activities which result in toxic effects, such as convulsions [5], injury to the cardiovascular and skeletal systems [6,7], and suppression of food consumption and body weight gain [8,9] in SE-treated animals. Furthermore, it was shown to act as a reproductive endocrine disruptor with anti-estrogenic activity to the female reproductive system [10,11].

A recent study showed a prominent effect of SE on male reproductive system in zebrafish (Danio rerio) through modulating gene transcription, reducing sex hormone levels, and impairing testis structure [12]. The study also indicated that the effective concentrations of SE identified to alter sex hormones and gene expression were lower than the reported environmentally relevant concentrations [13].

Turmeric (TU), also known as *Curcuma longa* L., is a rhizomatous herbaceous perennial flowering plant of the ginger family, Zingiberaceae that is recognized as a biologically active natural substance. It has high absorption rate, an exceedingly low degree of toxicity [14], and antioxidants abilities exerting hyperactive oxygen free radical scavenging effects and increased intracellular glutathione acclaim, thereby protecting lipid peroxidation1 [5–17]. It is also used as a spice, coloring, and flavoring and in

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traditional medicines [18]. TU and its extracts have various beneficial properties on human health, such as anti-inflammatory, anti-carcinogenic, anti-tumoral, antiviral, anti-fungal, anti-parasitic, anti-mutagen, anti-infectious, anti-hepatotoxic, and antioxidant compound activities [19–23]. The main active ingredient is curcuminoid [1,7-bis(4-hydroxy-3-methoxyphenyl)-1,6-heptadiene-3,5-dione] [24,25]. Defensive effects of curcumin, the naturally occurring chemical compound found in the spice TU, on the testis have been detected previously [26,27]. In addition, many studies have established the antioxidant properties of TU against oxidative stress [28,29] and have shown protective effect on male reproductive system against chromium-induced oxidative damage [30].

Caspases, a family of protease enzymes, playing essential roles in the machinery response of programmed cell death (i.e., apoptosis) and inflammation. Caspase-3 is the major effector caspase involved in apoptotic pathways [31], while Bax is a proapoptotic member of Bcl-2 protein subfamily and plays a fundamental role in the apoptosis process [32]. Genes and proteins can be either pro-apoptotic (Bax, BAD, Bak, and Bok) or anti-apoptotic (Bcl-2, Bcl-xL, and Bcl-w). These genes bond with the Bcl-2 protein, resulting in either a pro- or anti-apoptosis function [33].

This study aims to study the effects of SE on male reproductive system (i.e., testis) of juvenile Wistar rats and report hormonal levels of total triiodothyronine (T3), total thyroxine (T4), and testosterone as well as the testicular histological and immunohistochemical changes.

Materials and Methods

Drug sources

SE (S2201 ≥ 99%) was purchased from Sigma-Aldrich (St. Louis, MO). TU 500-mg tablets were purchased from GNC Nutrition Corp. (Pittsburgh, PA).

Animals and experimental design

After 1 week of acclimatization, 40 juvenile male Wistar rats (*Rattus norvegicus*), 4-week-old (90–95 g) were divided into four groups (10 animals/treatment) and kept in polypropylene cages with wood chip bedding and water and food available *ad libitum*. Animals were under a 12-h light/dark cycle and temperature of 22°C–24°C. Experimental and animal protocols were approved in agreement with the Canadian council on animal care [34]. Male rats of the first group were considered the control group, administered with distilled water. Animals of the second group received TU powder (300 mg/kg bw/day) orally with the help of a feeding cannula for 28 days [35], while the animals in the third group received oral administration of SE (75 mg/kg bw/day) for 28 days [7]. Rats belonging to the fourth group received SE then after 1 hour, TU was administered orally. This treatment was performed daily for 28 days.

Body weights

Animals were individually weighed by means of a Meopta sensitive balance. Whole body weights were recorded to the nearest 1 mg to determine weekly changes.

Testis weights

Fresh left and right testes from autopsied rats were blotted dry and subsequently weighted. Absolute testis weights were recorded to the nearest 0.1 mg using an electric balance.

Blood sampling and biochemical analyses

Blood samples were collected from the heart by sterile syringes and left to clot, centrifuged to separate the serum at 3,000 rpm for 15 minutes. Samples were directly frozen for later biochemical analyses at −10°C. Serum level of testosterone, T3, and T4 were measured using VIDAS® fertility panel and VIDAS® thyroid panel, respectively, from BioMérieux kits (Marcy-l'Étoile, France) according to the manufacturer's protocol.

Histological examinations

Testicular tissues were removed from sacrificed juvenile animals, weighed, fixed in 10% neutral buffer solution, and processed to get paraffin wax blocks according to the standard procedure (sectioned at 4 μm thickness). Blocks were then further deparaffinized with xylol and histologically observed under light microscope using hematoxylin and eosin (H&E) staining.

Immunohistochemical investigations

The advantage of the immunohistochemical methods is the ability to show protein location inside the cells indicated by color changing, which exploits the principle of antibodies binding specifically to antigens. Sections from paraffin blocks with 4 μm were cut and collected on glass positive slides. After

de-waxing and using the protocol according to the manufactured instructions, proteins were demonstrated by immunohistochemistry. Positive slides were overnight incubated at 4°C with the primary antibody of anti-caspase-3 (ab4051) and anti-Bax (ab7977); washed and stained with a streptavidin-peroxidase detection system. The antibodies were purchased from ABCAM (Cambridge, United Kingdom). By omitting the primary antibodies, negative control was obtained.

Image analysis

Positive slides of primary antibody were quantified for both caspase-3 and Bax using web application ImmunoRatio software (version 1.0c) [36]. The percentage of immune-reactive cells (stained brown) and the percentage of area over all tissue cells (labeling index) were calculated and statistically analyzed. Utilizing this web-based software reflected the immune-interaction sites with immune tinctures that indicates the occurrence of cell death and represented graphically the proportion of damaged cells.

Statistical analysis

Data were represented as mean ± standard error and analyzed using one-way analysis of variance by the SPSS for Windows software, version 16.0 (SPSS, Chicago, IL) to compare all groups. Once a significant *F* test was obtained, a *post hoc*-least significant difference analysis was performed with the significance level of $p < 0.05$.

Results and Discussion

Growth rates and testis weight

Average body weight values were 95.03 and 95.38 g in both control and TU groups, respectively. The administration of SE caused a significant reduction ($p < 0.05$) in body weight of juveniles reaching 70.16 g. The administration of TU + SE reported a significant increase in body weight reported in juvenile male rats ($p < 0.05$) reached 85.59 g compared to SE-treated animals (Table 1). Average weight of both left and right testes were recorded as 1.65 and 2.01 g for control group, while they were 1.60 and 1.93 g for TU group. SE administration caused a decrease in left and right testes reaching 0.75 and 0.81 g, respectively. The administration of both SE and TU revealed a significant ($p < 0.05$) increase in both testes weight reached 1.58 g for left testis and 1.62 g for right testis compared to SE group.

These results from this study coincide with Maranghi et al. [7] that showed SE significantly decreased body weight gain in males even at the lowest dose (0, 40, 75, and 140 mg/kg bw/day) for 28 days. In addition, a study have shown that SE present at 0.125% in drinking water for a long time caused limitation in food and water intake of male mice and decreased body weight due to the impairment of fat deposition [9]. Many reports attributed the decrease in testicular weight mainly to loss and degeneration of germ cells, causing exerted pleiotropic effects during juvenile period [7,37,38].

Biochemical levels

The results indicated that rats treated with TU only delineated a non-significant change in serum levels of T3, T4, and testosterone compared to control group (Table 2). Conversely, the administration of SE to juvenile male rats induced a significant decrease ($p < 0.05$) in T3, T4, and testicular testosterone production levels compared to control and TU groups. Such decrease is not an uncommon effect of SE treatment as it has been observed in previous studies. The decrease in hormone levels is attributed to a number of factors, including the inhibition of the total number of Leydig cells that cause strong decrease in the expression of testosterone [39], augmentation in degenerated germ cells at different stages of development [40], and inhibition in serum insulin and luteinizing hormone levels, which may have a role in the impairment of Leydig cell function [41].

Furthermore, the decrease may also be related to the decreased number in Sertoli cells and the damaged Leydig cells that appear in the testicular tissue sections analysis. Sertoli cells play a central role in testis differentiation and proliferate more actively before birth and, in rats, proliferation extends 2–3 weeks postnatal [42,43] where they determine the number of Sertoli cells in adult testis [44] and hence, fertility. While T3 and T4 influence the duration of Sertoli cells proliferation, their deficiency causes failure Leydig cells differentiation, retard Sertoli cell maturation, and decrease T3, T4, and testosterone levels [45].

On the other hand, results in this study have shown the levels of T3, T4, and testosterone to increase in the SE and TU group compared to SE group (Table 2). This restoration concurs with a previous study that showed TU restoring testis functions by reducing cellular stress and acting on the key mediators of the apoptotic cell death, such as Jun N-terminal kinases and p38 [46].

Table 1. Body and testes weight in control and different experimental groups of juvenile rats.

Experimental groups	Control group	TU	SE	TU + SE
Body weight (g)	95.03 ± 0.68	95.38 ± 0.62	70.16 ± 1.91^{ab}	85.59 ± 1.31^{abc}
Left testis weight (g)	1.65 ± 0.04	1.60 ± 0.04	0.75 ± 0.03^{ab}	1.58 ± 0.03^{c}
Right testis weight (g)	2.01 ± 0.03	1.93 ± 0.04	0.81 ± 0.03^{ab}	1.62 ± 0.07^{c}

Values are presented in mean ± standard error with significant difference ($p < 0.05$) compared to; a: control group. b: TU group. c: SE group.

Table 2. Serum levels of glucose, testosterone, total triiodothyronine (T3), and total thyroxine (T4) in different groups of juvenile rats.

Experimental groups	Control group	TU	SE	TU + SE
T3 (pg/ml)	9.06 ± 0.35	9.63 ± 0.29	3.52 ± 0.32^{ab}	6.19 ± 0.44^{abc}
T4 (pmol/l)	14.43 ± 0.26	15.38 ± 0.27	8.53 ± 0.31^{ab}	11.64 ± 0.23^{abc}
Testosterone (ng/ml)	4.26 ± 0.23	4.46 ± 0.23	1.7 ± 0.17^{ab}	3.93 ± 1.01^{c}

Values are presented in mean ± standard error with significant difference ($p < 0.05$) compared to; a: control group. b: TU group. c: SE group.

Histological examinations

Histological analysis showed the testis parenchyma in the control group to have normal profile, including many seminiferous tubules of varying sizes; each is surrounded by an outer thin layer of connective tissue, lined with germinal epithelium that surrounds a central lumen and germinal epithelial layer consists of Sertoli cells and spermatogenic cells (Fig. 1A). The TU group also showed similar normality found in control group with normal spermatogenesis pattern of the seminiferous tubules and well-developed sperms represented (Fig. 1B). In contrast to such results, the treated SE group revealed the disturbance of seminiferous tubules arrangement, as zigzag edges tubule and degenerated intertubular tissue appeared (Fig. 2A). Complete tubular depletion and spermatogenesis arrest, congested hyalinized wall peripheral blood capillary, spermatogenic cells could not transform into sperm, and marked apical sloughing or shedding were observed (Fig. 2B). Moreover, acidophilic hyaline material was available in the greater part of the interstitial spaces and interstitial vacuolation, which could be attributed to abundance lymphatic exudates from degenerative lymphatic vessels or to an expansion in vascular [38,47] and/or to the expanded action of Leydig cells, thus expanded the steroid content [48].

These seminiferous tubules could also be observed to have widened interstitial space in some areas, the germinal layer has a few spermatogenic cells with vacuolar cytoplasm and darkly stained nuclei (Fig. 2C). In addition, spermatogenesis arrest

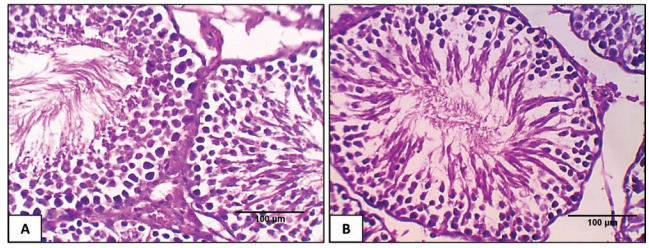

Figure 1. Sections from testicular tissue. (A) Control group showing normal testicular architecture with an orderly arrangement of differentiating spermatogenic, Sertoli cells in seminiferous tubules, and Leydig cells in the interstitial space. (B) TU treated group showing results similar to the control group.

and dilated congested intertubular vessel and atrophied seminiferous tubules were observed (Fig. 2C). The SE group testicular tissue also exhibited hyaline material with vacuolated intertubular connective tissue, influenced Sertoli cells instigated changes or abatements in seminiferous tubule liquid discharge, distribution of few Leydig cells, spermatogenesis arrest, congested peripheral blood capillary, germinal epithelium dissociation, and numerous dark nuclei (Fig. 2D). This is probably due to the consequence of metabolic unsettling influence in these cells and changes in their morphology and that the hyaline material is made out of deposit plasma proteins [49]. A spillage of plasma proteins over the endothelium may become trapped with union of arrangement of cellar film segments by the smooth muscle cells lined blood vessels. These results were confirmed by the previous studies where they reported markedly distorted testicular parenchyma

and seminiferous tubules with irregular shape, disorganized epithelium, wide lamina, and clear shedding of cells due to seminiferous tubule liquid discharge [37,38,50–53].

Reactive oxygen species (ROS), with excessive generation can originate oxidative stress, caused major impact on many male reproductive processes, including fertilization, acrosome reaction, hyperactivation, motility, and capacitation [54,55]. Given the particular vulnerability of mammalian spermatozoan to lipid peroxidation due to their high lipid composition [56], a rise in ROS levels will affect the sperm structure and function [57]. Furthermore, a study on human showed that toxins released from structural materials or industrial products accumulate in the human body and increase ROS production in the testes, negatively impacting the sperm structure and function [58]. In accordance with this, the mutagenicity of SE, being a chemical compound, have shown to be associated with the generation of ROS and that it acts as a strong inhibitor of ROS scavengers, such as superoxide dismutase and glutathione peroxidase [59–61].

On the other hand, treating with TU in the SE and TU group showed illustrated marked improvements compared to SE group with nearly normal pattern of seminiferous tubules and wide intertubular space (Fig. 3A), rearrangement layered of spermatogenesis cells and necrotic intertubular connective tissue area along side by side with regeneration of testicular layers (Fig. 3B and C). These results coincide with Sharaf [62] where TU have been shown to scavenge free radicals and thus, act as an antioxidant. Its role as an antioxidant may be due to its ability to down-regulate free radicals formation and up-regulate cell membrane permeability and transport functions [63].

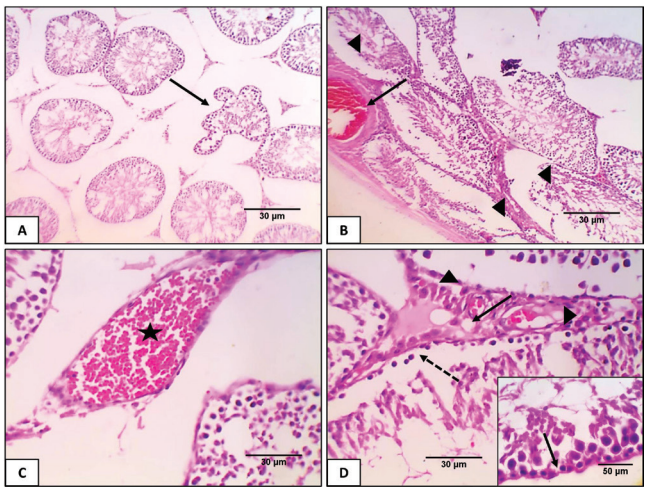

Figure 2. Sections from testicular tissue of the SE group showing (A) Zigzag edges of seminiferous tubule and degenerated intertubular tissue (arrow). (B) Complete tubular depletion (arrowheads), tissue arrest, and congested hailinzed wall peripheral capillary (arrow). (C) Dilated and congested intertubular vessel (star) and seminiferous tubules atrophy. (D) Spermatogenesis arrest with separated area (dotted arrow), hyaline material with vacuolated intertubular matrix (arrow), and few Leydig cells (arrowheads). Insertion. Erosion in the spermatogenic cell layers (arrow) and numerous dark nuclei.

Immunohistochemical studies

To determine the degree of tissue damage and histological cell death at the level of nucleus defects, immunohistochemical techniques were implemented. Immunohistochemical staining of caspase-3 showed no evidence in the control and TU groups (Fig. 4A and B), positive reaction (brown chromogenic site) in SE group (Fig. 4C), and fewer reactive sites in SE and TU group (Fig. 4D). The immunohistochemistry analyses revealed a significant increase distribution of caspase-3 positive immune-reaction in the cytoplasm of SE group compared by control. SE and TU treated group showed a decline in immune-reaction response of caspase-3 indicating up-regulation of the protein (Fig. 5).

ROS are important in mediating apoptosis by inducing cytochrome c and caspases 9 and 3, which in turn result in a high frequency of single- and double-stranded DNA strand breaks [64].

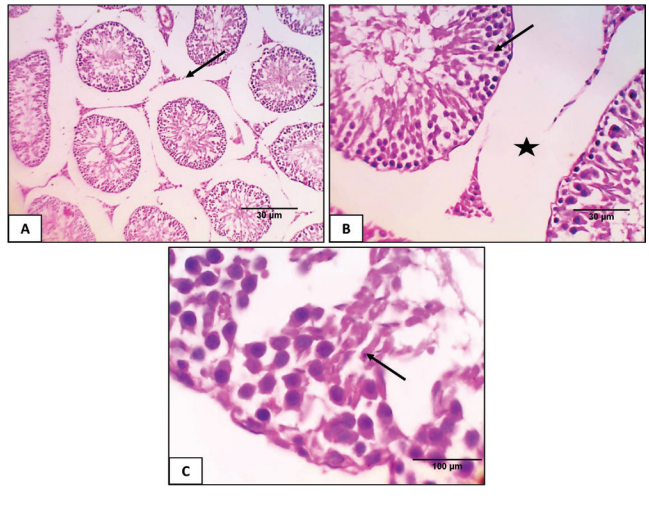

Figure 3. Sections from testicular tissue of the TU + SE group showing (A) nearly normal pattern of seminiferous tubules with wide intertubular space (arrow). (B) Rearranged layered of spermatogenesis cells (arrow) and necrotic intertubular connective tissue area (star). (C) Regeneration of testicular layers.

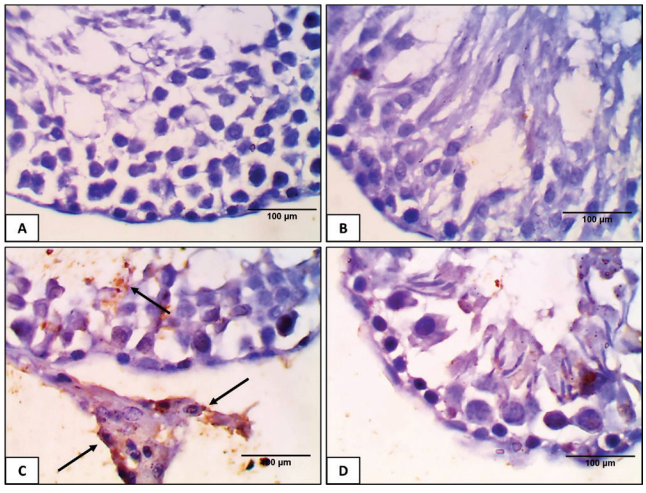

Figure 4. Sections from testicular tissue in Immunohistochemical staining for caspase-3. (A) Control group showing no evidence of caspase-3 reaction. (B) TU group showing similar results to control group. (C) SE group showing positive reaction to caspase-3 (brown chromogenic sites) compared to control and TU groups. (D) TU and SE group showing less caspase-3-positive reaction cells.

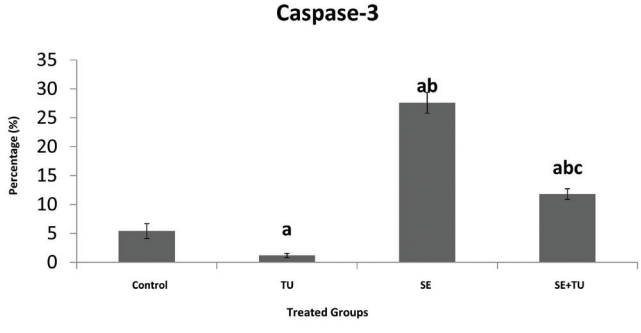

Figure 5. Chart illustrating the percentage of caspase-3 immune reaction in testicular tissue sections of all groups. The values are presented as mean ± standard error and significance ($p < 0.05$) compared to (a) control group, (b) TU group, and (c) SE group.

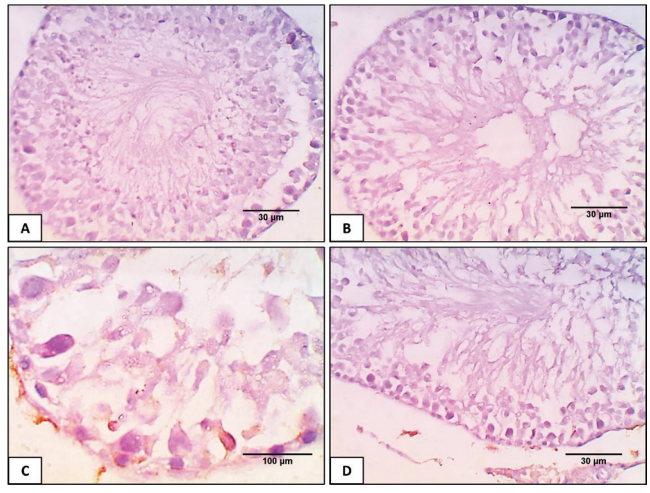

Figure 6. Sections from testicular tissue in immunohistochemical staining for Bax. (A) Control group showing no evidence of Bax reaction. (B) TU group showing similar results to control group. (C) SE group showing positive reaction to Bax (brown chromogenic sites) compared to control and TU groups. (D) TU and SE group showing less Bax-positive reaction cells.

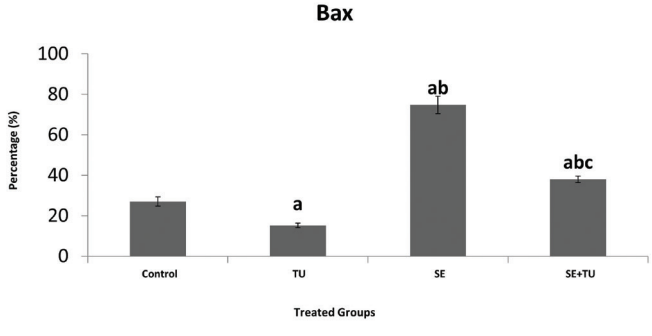

Figure 7. Chart illustrating the percentage of Bax immune reaction in testicular tissue sections of all groups. The values are presented as mean ± standard error and significance ($p < 0.05$) compared to (a) control group, (b) TU group, and (c) SE group.

Moreover, staining reaction of pro-apoptotic (Bax) in cell nucleus showed no evidence in the control and TU groups (Fig. 6A and B), intensive positive reaction (brown chromogenic site) in SE group (Fig. 6C), and fewer reactive sites in SE and TU group (Fig. 6D).

These investigations were authenticated with image analysis, which illustrated few distributions of protein level in both control and TU groups and elevation in the SE group compared to control and SE and TU groups (Fig. 7).

Similar response of increased caspase-3 and Bax distribution were reported previously [65], which indicated elevation apoptotic cells in SE group. Some studies attributed such response to external stimuli, such as drugs radiation, temperature, and ecological poisons, which increment the levels of apoptosis in germ cells [66]. A study have shown that testicular tissue of SE-treated animals is manifested with numerous apoptotic lesions and positive reaction of Fas-Ligand distribution, which indicate similar results to caspase-3 and Bax distribution due to SE treatment [53]. In addition, TU has the ability to diminish the development of tumor cells have been shown through control of numerous cellular pathways, including cell multiplication

pathway, caspase enactment pathway, tumor silencer pathway, and protein kinase pathway [67].

Conclusion

The present study on the effect of SE administration on testicular tissue of juvenile rats discovered that testicular cells are inclined to undergo apoptosis after exposure to SE and that TU treatment can mitigate such deleterious effects with its anti-apoptotic properties. In addition, the destructive effect of SE is shown not only on the testicular tissue but also on the cellular level, including the nuclear defects. Therefore, the present study strongly recommends that SE should be banned from using in the manufacturing bottles of babies and young people food, taken into consideration that the baby food packed in glass jars has a life of 3 years.

Abbreviations

SE = semicarbazide;
TU = turmeric;
T3 = total triiodothyronine;
T4 = total thyroxine;
H&E = hematoxylin and eosin;
ROS = reactive oxygen species.

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Conflict of Interest

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

Authors contributions

Amal A. E. Ibrahim, Amany A. Osman, Mona A. F. Nasser, and Mona R. Alshathly conceived and designed the study; Amal A. E. Ibrahim and Amany A. Osman performed experiments; Mona A. F. Nasser and Mona R. Alshathly performed the analysis; all authors contributed to the writing of the manuscript; and Mona R. Alshathly approved the final version.

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LETTER TO THE EDITOR

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Drugs and dietary supplements with unproven effects in research and practice: Part 2

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ABSTRACT

Several examples are discussed in this review, where substances without proven effects were proposed for practical use. The following is discussed here: generalizations of the hormesis concept and its use in support of homeopathy; phytoestrogens and soy products possibly having feminizing effects; glycosaminoglycans for the treatment of osteoarthritis and possibilities of their replacement by diet modifications; flavonoids recommended for the treatment of chronic venous insufficiency and varicose veins; acetylcysteine as a mucolytic agent and its questionable efficiency, especially by an oral intake; stem cells and cell therapies. In conclusion, placebo therapies can be beneficial and ethically justifiable, but it is not a sufficient reason to publish biased information. Importantly, placebo must be devoid of adverse effects, otherwise, it is named pseudo-placebo. Therapeutic methods with unproven effects should be tested in high-quality research shielded from the funding bias. Patients participating in such research must be treated free of charge. As for animal experiments, they should be performed by integer researchers not influenced by conflicts of interest. The potential outcomes of some of these issues are not entirely clear, and the arguments provided here can initiate a constructive discussion.

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Introduction

This is a continuation of the preceding review about substances with unproven or questionable effects, some of them directly or indirectly represented as evidence-based medications [1]. In Russia, the marketing of placebos under the guise of evidence-based medications is known to occur [2,3]. Several examples are discussed in this review, where drugs and dietary supplements with unproven effects and unclear action mechanisms are presented as evidence-based medications. The conclusions of this review are partly based on theoretic considerations. In conditions of abundant literature with declared and non-declared conflicts of interest, theoretic considerations gain importance [4,5]. When theories are adopted by many different researchers, they can build up a coherent body of work [4], whereas wrong concepts become easier to recognize. Physicians with insufficient theoretic education might prescribe advertised medications without pondering on physiological mechanisms [6].

Not only questionable data have been published but also theoretic concepts were construed. An example is a hormesis—a concept of biphasic dose-response to various pharmacological and toxicological stimuli; typically, low-dose exposures induce a beneficial response while higher doses cause toxicity [7]. Theoretically, hormesis as a general principle is conceivable only for factors that are present in the natural environment, having induced an evolutionary adaptation so that a deviation in either direction from an optimum would be harmful [8]. Examples are different kinds of stress, numerous chemical substances, and elements [7,9]. However, there are no reasons to expect hormetic (biphasic) dose-responses *a priori* for factors that are absent in the natural environment.

A response to a pharmacological agent generally tends to increase with the increasing dose and concentration. However, homeopathic remedies can be extremely diluted. Homeopathy claims a curative action for small drug doses, of which high doses

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would cause symptoms similar to those the patient has. Homeopathy has never been grounded on scientific evidence [10]. Nonetheless, homeopathic medications have been proposed, patented, and used in diverse diseases, e.g., tuberculosis, acute pneumonia, viral infections, and myopia [11–14]. In particular, it is precarious when homeopathic medications are delivered by invasive methods, e.g., intraarticular injections [15]. If homeopaths have useful empirical knowledge, it should be discussed in the professional literature and tested by scientific methods. Suggestions that homeopathy is based on hormesis create an illusion that it employs a scientific concept.

Some agents can have cumulative effects or act synergistically with other noxious factors, e.g., on cells with a limited ability of cellular regeneration, such as cardiomyocytes or neurons. It can be of particular importance in conditions when such cells are pre-damaged so that even a mild additional impact would act according to a no-threshold pattern without hormesis. Under such circumstances, which are not uncommon especially in gerontology, hormesis concept can be unsafe if used in clinical thinking [16]. For example, it is obviously not indicated to ingest small amounts of ethanol, a known hormetic agent, by a patient with hepatic failure. Nonetheless, certain publications, describing hormesis as a general biological principle [17,18], can be cited in support of homeopathy and placebos as inexpensive substitutes of evidence-based medications. It should be stressed that all clinically significant effects, hormetic or not, must be tested according to the principles of evidence-based medicine.

Focused Review

Phytoestrogens and soy products

Phytoestrogens (PhE) are plant-derived substances with structural similarity to estradiol [19,20]. The most extensively studied PhE are isoflavones and coumestans. Isoflavones are most abundant in soybeans. Some other plants also contain PhE, in particular, red clover. PhE are used for compensation of estrogen deficiency in menopause; however, their estrogenic potential does not prevent the use of soy in infant formulas, other foodstuffs, and pediatric parenteral nutrition.

The consumption of PhE and soy foods has been associated with health benefits [21]; however, the potential adverse effects on the reproductive and endocrine system may be underappreciated [19,22]. Some epidemiological studies suggest that dietary intake of PhE contributes to a decreased incidence of postmenopausal cardiovascular and thromboembolic events [23], while PhE are significantly more effective than placebo in reducing the frequency and severity of hot flashes [24]. However, the evidence from observational studies and randomized trials has been generally inconclusive [25,26]. Several reviews concluded that there are no reliable arguments in favor of PhE efficacy against menopausal symptoms [27,28] and that current evidence does not generally support their use [29]. The efficacy of PhE against vasomotor symptoms has failed the test of randomized clinical trials [30]; "Efficacy of PhE on menopausal vasomotor symptoms is similar to placebo" [31]; "Definite conclusion on possible beneficial health effects of PhE cannot be made" [20], etc. The analysis of earlier findings from the enrichment of the diet with soy protein has failed to confirm beneficial cardiovascular effects by way of lipid-lowering, vasodilatation, or lipoprotein oxidation [32]. In particular, there are little data to support the claim that PhE protect against menopausal osteoporosis [20,33–36]; although the matter is controversial and favorable effects have been reported [37,38] (Table 1). For example, the following statement is questionable: "Comparative assessment showed no significant differences between the effectiveness of the hormone therapy and the PhE used in the study, in terms of effects on bone mineral density and bone resorption" [38] as the hormonal activity of the PhE is known to be much lower than that of estradiol and norethisterone acetate used in the study [38]. The European Food Safety Authority concluded that the available evidence was not sufficient to establish a relationship between the maintenance of bone mineral density and the consumption of soy isoflavones [20].

The use of the PhE as an alternative for the hormone replacement therapy is not advocated also because of insufficient and conflicting data about safety [46]. Sporadic reports on adverse effects and interactions with other medications are appearing [47]. Moreover, soy is one of the most allergenic foods; so that for some people, it is essential to avoid it [20,48]. The conventional menopausal hormone therapy remains the only treatment that consistently had a greater effect than placebo in published controlled trials [49]. The majority of high-quality studies demonstrated no clear benefit and some potential for harm, further research being necessary to make recommendations [49].

Table 1. Trials on phytoestrogens in menopause with measurable endpoints.

Title: Effects of phytoestrogen-rich diets on bone turnover in postmenopausal women Characteristics: Randomized clinical trial, 300 participants, 2002–2004 Conclusion: Soy isoflavone did not prevent bone loss in healthy early postmenopausal women [39].
Title: Bone sparing effects of soy phytoestrogens in menopause Characteristics: Randomized clinical trial, 248 participants, 2003–2009 Conclusion: The daily administration of 200 mg of soy isoflavones for 2 years did not prevent bone loss or menopausal symptoms [40].
Title: CVD risk and health in postmenopausal phytoestrogen users Characteristics: Randomized clinical trial, 210 participants, 1997–2004 Conclusion: Dietary isoflavone consumption may be protective against bone loss in postmenopausal women through a reduction in bone resorption [41].
Title: Third year evaluation on genistein efficacy and safety Characteristics: Observational cohort study, 138 participants, 2005–2006 Conclusion: Genistein exhibited a promising safety profile with positive effects on bone formation in a cohort of osteopenic, postmenopausal women [42].
Title: Effect of genistein in women with metabolic syndrome Characteristics: Randomized clinical trial, 120 participants, 2007–2011 Conclusion: Genistein has positive effects on bone mineral density in osteopenic postmenopausal women [43].
Title: Women's isoflavone soy Health (WISH) trial Characteristics: Randomized clinical trial, 350 participants, 2004–2009 Conclusion: Isoflavone soy protein (ISP) did not significantly reduce subclinical atherosclerosis progression in postmenopausal women. ISP may reduce subclinical atherosclerosis in healthy women at low risk for cardiovascular disease who were <5 years postmenopausal [44].
Title: Safety and effectiveness of soy phytoestrogens to prevent bone loss (OPUS) Characteristics: Randomized clinical trial, 403 participants, 2001–2006. Conclusion: Soy hypocotyl isoflavones reduce whole-body bone loss but does not slow bone loss at common fracture sites in healthy postmenopausal women [45].

The theoretical basis for the use of PhE for menopausal hormone replacement appears doubtful. The biological action of estrogens is mediated by receptors. It appears unclear, why accidental plant-derived analogs must be used instead of natural or synthetic hormones. Another reasonable question: "Why should soy or red clover products containing isoflavone be recommended, if the positive effects are only negligible but the adverse effects serious?" [50]. Moreover, commercial PhE preparations sometimes contain a mixture of ingredients of obscure nature. Such mixtures can exert unpredictable effects, depending on their composition and the patient's condition [51]. The concept of the PhE as a "natural and safe" alternative to estrogens [30] is unfounded: these substances are in fact less natural for humans than endogenous hormones. The following controversies should be stressed: PhE are used for compensation of estrogen deficiency in the menopause; and, at the same time, their estrogenic potential does not prevent from the use of soy in infant formulas and other foodstuffs. Moreover, the use of soy as animal fodder may result in accumulation of the PhE and their active metabolites, such as equol in meat and other animal products. Equol, a metabolite of the PhE with a relatively high estrogenic potential, is produced by intestinal bacteria in sheep, cows, pigs, and domestic fowl [52,53].

Adverse effects associated with the intake of soy have been reviewed [19,54–56]. Perturbations of the reproductive health and feminizing effects in men are regarded to be rare and mild [54] but they might be statistically detectable in large populations. It has been reported about abnormal uterine bleedings in women consuming soy products, subtle gender-related behavior changes in girls, and gynecomastia in a man [19,57,58]. A cross-sectional study of 11,688 women showed that a high intake of isoflavones was related to an increased risk of never becoming pregnant [59]. Hormonal effects of the PhE may lead to fertility problems possibly due to the impact on the menstrual cycle, oocyte quality, and endometrial receptivity [55]. An association between soy exposure and early menarche was reported [60] although there was a contradicting report [54]. Animal data demonstrate that soy isoflavones, at doses and plasma levels possible in humans including infants, can influence neuroendocrine pathways in both sexes. Relevant doses of the PhE have an impact on the differentiation of ovaries and fertility in animals [19,61,62]. Alterations of male sexual development

and deficits of sexual behavior were noticed in rats [63]. Moreover, certain PhE, e.g., genistein can exert androgenic effects [64], which is not surprising as PhE are plant substances with accidental similarity to human hormones. It was suggested that isoflavones are selective estrogen receptor modulators and, as such, are different from estrogens. It is doubtful, however, whether such modulation, also named "endocrine-disrupting properties of soya" [19], is favorable for infants receiving soy formulas and for other consumers of soy products [19,65]. In addition, soy-based oil emulsions are causative factors of cholestasis related to pediatric parenteral nutrition; further details and references are in [66].

Another contradiction: it was stated that "findings from a recently published meta-analysis" and subsequently published studies show that neither isoflavone supplements nor isoflavone-rich soy affects total or free testosterone levels. Similarly, there is essentially no evidence from the nine identified clinical studies that isoflavone exposure affects circulating estrogen levels in men" [67]. In a case report on gynecomastia associated with soy consumption by a man, it was noted: "After he discontinued drinking soy milk … his estradiol concentration slowly returned to normal" [58]. Statements of this kind, for example, that there was "no conclusive interaction between soy or isoflavone intake and free testosterone concentrations" and "a systematic review of the literature showed that effect of soy on sex hormones in pre- and post-menopausal women had very small" [54] are potentially misleading because PhE, being estrogen analogs, exert hormonal effects on their own, not necessarily influencing the levels of the endogenous hormones.

In Russian-language literature, the PhE are sometimes promoted using misquoting of foreign publications; examples are in [68]. Supposed anti-atherogenic efficiency of certain PhE has been corroborated by experiments with cell cultures, where the ability of serum to induce accumulation of cholesterol in cultured cells was interpreted as an indicator of atherogenicity [69,70]. The reliability of these experiments has been questioned; however, publications of that kind are continued without references to the published criticism [2,3]. All said numerous studies positively characterizing PhE cannot be dismissed (Table 1). It was not the purpose of this review to pass final judgments on the efficiency and safety of the PhE. The aim was to point out that PhE are used to compensate for estrogen deficiency in menopause, but their estrogenic potential does not prevent from the use of soy in infant formulas and other foodstuffs. As mentioned above, the feminizing effect of soy products may be subtle, detectable only statistically in large populations. This matter should be clarified by independent research.

Glycosaminoglycans for the treatment of osteoarthritis

Chondroitin (Ch) is a glycosaminoglycan and glucosamine (Ga) is an aminosaccharide acting as a substrate for the biosynthesis of glycosaminoglycans. Ch undergoes hydrolysis in the intestine; being administered orally, it can be regarded as a source of precursors for glycosaminoglycans. Hyaluronic acid (HA) is a glycosaminoglycan used for intra-articular injections. These substances are named chondroprotectives and applied for the treatment of osteoarthritis. The oral preparations have been discussed as Symptomatic Slow-Acting Drugs in Osteoarthritis [71]. This term seems to be suboptimal: oral glycosaminoglycans and their precursors are not sensu stricto symptomatic as they are aimed primarily not to alleviate symptoms but to compensate for a supposed deficiency of constituents/precursors of cartilage or synovial fluid. The evidence in favor of their chondroprotective effectiveness is conflicting. Many studies have been sponsored by the industry. There is skepticism in the scientific community [72]. For example, a meta-analysis concluded that "Ch, Ga, and their combination do not have a clinically relevant effect on perceived joint pain or on joint space narrowing" [73]. Another key remark: "Given that there is an effect, understanding the biochemical basis of this effect might lead to more useful supplements" [74]. However, the biochemical basis is largely unclear. Glycosaminoglycans and their precursors are not irreplaceable; they are produced by the body, also in vegetarians who consume no immediate precursors. It appears doubtful that oral supplementation of Ch or Ga can shift the balance between synthesis and degradation in the whole body so that it would be significant for joint cartilage. Furthermore, the sources such as shellfish chitin and fungi for Ga, cartilage from mammals, birds, or fish for Ch as well as contaminants can impart undesirable properties to the preparations [75,76]. Pain, stiffness, and other criteria of efficacy are largely subjective, which means that a great part of the treatment successes of osteoarthritis can be attributed to the placebo effect [77–80]. It is known that pain measurements in clinical trials are difficult, possibly contributing to the exaggeration of treatment effects. There are

many studies and reviews reporting the efficiency of chondroprotectives compared to placebo, but reliability is often questionable due to declared or non-declared conflicts of interest. Quality of research and possible influence by the industry must be taken into account defining inclusion criteria for studies into meta-analyses and reviews. According to the high-quality trial GAIT, Ch or Ga alone or in combination did not reduce pain in patients with osteoarthritis of the knee. Overall, differences between the agents and placebo were small. However, exploratory analyses suggested that the combination Ch + Ga may be effective in the subgroup with moderate-to-severe knee pain. Treatment with Ch was associated with a significant decrease in the incidence of joint swelling and effusion [81]. Admittedly, the effects were mostly on subjective symptoms where the placebo effect comes into question. Potential bias related to inadequate masking of study agents was discussed [81]. In any case, if the combination Ch + Ga alleviates the suffering of arthritis patients, this alone may justify its clinical use.

In regard to the intra-articular injections of HA, a meta-analysis concluded that "currently available evidence suggests that intra-articular GA is not clinically effective" [82]. Another meta-analysis and systematic review concluded that in patients with knee osteoarthritis, intra-articular HA is associated with a small, clinically irrelevant benefit, and an increased risk of adverse events [77,83]. The evidence remains inconsistent and controversial [84]. Action mechanisms of intra-articular HA are hardly understandable including the "lubrication at the joint surfaces" [85], i.e., viscosupplementation. Viscosity changes after HA injections can be measured (e.g., adding HA to cadaverous synovial fluid) or approximately calculated, knowing the viscosity of the synovial fluid, of injected solution, and corresponding volumes. Both pre- and post-treatment viscosity indicators were reported to be within the range of normal values [86]. In any case, the lubrication effect cannot last long: no explanation has been found for the discrepancy between the short intra-articular half-life of injected HA and the reported duration of the clinical carry-over effect. The rheological effect of exogenous HA in the joint is supposed to last less than 1 day [87]. For example, the intra-articular half-life of Hyalgan (sodium hyaluronate) is about 17 hours; the low molecular weight component of Synvisc (Hylan G-F 20 constituting about 90% of the preparation) has a half-life of 1.5 days; the minor component with a higher molecular weight has a half-life of 8.8 days [86]. By contrast, the carry-over effect after the treatment cessation lasted from 3 months with oral chondroprotectives to 6–9 months with intra-articular injections [88]. The half-life of an HA preparation with artificial cross-linking was reportedly up to 4 weeks [89]; but again, there are no reasons to expect a much longer carry-over effect. A short-term functional improvement due to the viscosupplementation may reinforce the placebo effect by the mechanism of conditioning. It is known that invasive procedures can have a pronounced placebo effect. By definition, a placebo must be devoid of adverse effects; otherwise, it is named pseudo-placebo [90]. In particular, the intra-articular therapy of hip osteoarthritis is associated with adverse effects due to the proximity of important anatomical structures [91].

HA is a polymer; according to the law of mass action, its local enrichment would displace the chemical equilibrium toward low-molecular precursors, i.e., reduction of viscosity. Therefore, suppositions about enhanced biosynthesis of endogenous HA [71,92] after injections of the same substance are unfounded. As for molecular mechanisms studied *in vitro*, their clinical relevance is questionable, among others, because of generally higher concentrations of tested substances *in vitro* than *in vivo*. Besides, Ch, Ga, and HA have primarily been chosen for a supplementation therapy; a probability of their specific, e.g., anti-inflammatory action, inhibition of chondrodegenerative enzymes, or pain mediators, etc. [92–94], is *a priori* the same as for substances taken at random.

In Russia, Ch, Ga, and HA are named chondroprotectors. These drugs are prescribed to osteoarthritis patients including elderly people with low incomes. Many patients purchase drugs for prolonged use [95,96]. For example, the annual therapy with Theraflex® (Ch+Ga), broadly advertised in Russia, costs today at least 10,000 rubles (160 US dollars). The average pension in Russia was 2300 dollars per year in 2016 [97]. In the author's opinion, it would be more or less equivalent to recommend to osteoarthritis patients a diet rich in natural glycosaminoglycans: animal joints, chicken wings, etc. This idea is not new; it was discussed at conferences. To support the placebo effect, patients can be advised that such a diet would saturate their bodies with precursors of cartilage similarly to the pharmaceuticals. In this connection, it might be helpful to study the prevalence of osteoarthritis in vegetarians consuming neither glycosaminoglycans

nor their direct precursors. Effectiveness of dietary supplementation of natural glycosaminoglycans compared to Ch and Ga preparations can be tested in animals with osteoarthritis, particularly dogs, giving them food rich in cartilage. A recent review concluded that potential benefits from Ga and Ch in canine osteoarthritis can neither be confirmed nor denied [98]. Unfortunately, not only human but also animal studies are at risk of funding bias [98].

Flavonoids as venoactive drugs

This section is about flavonoids (Fl) used for the treatment of chronic venous insufficiency and varicose veins. Fl prevail among dietary polyphenols; they are found in many fruits, vegetables, and cereals [99,100]. In the past, some supposedly venoactive Fl (rutin, escin, and quercetin) were produced from medicinal plants. Today, the micronized purified flavonoid fraction (MPFF) consisting of 90% diosmin and 10% hesperidin is broadly used [101]. Diosmin is synthesized from hesperidin extracted from oranges [102]. In the U.S., preparations of Fl are classified as dietary supplements (medical food), and in some European countries—as drugs, which does not necessarily mean extensive use. In Scandinavia, drugs are rarely prescribed for the chronic venous disease [103,104]. In Spain, for certain phlebotonics (calcium dobesilate, chromocarbe, and naftazone), the indication for the use in chronic venous insufficiency has been withdrawn, while for several other phlebotonics, such as aminaftone, diosmine, hidrosmine, escin, and some rutosides, the use in exacerbations of chronic venous insufficiency has been limited to 2–3 months [105].

The following effects of venoactive Fl have been discussed: phlebotonic, anti-edematous, anti-inflammatory, and anti-oxidative. The action mechanisms are neither well established [102,105,106] nor clearly understandable. Smooth muscles (SM) of large and medium-sized veins have no appreciable tone and do not relax under the impact of vasodilators [107]. Lumina of collapsed veins are slit-like, circular SM bundles alternating with fibrous tissue. In post-thrombotic syndrome and varicose veins, where Fl are recommended, veins are distended, SM being atrophic and replaced by connective tissue [108,109]. This is against any significant phlebotonic effect of Fl; in particular, its durability is doubtful, which pertains also to a supposed potentiation of the norepinephrine action [102,107,109]. At the same time, there were reports on the inhibition by quercetin of norepinephrine-induced vascular contraction [110]. The vasoconstrictive effect of norepinephrine is transient; its blood concentration fluctuates, e.g., in stress, whereas Fl are supposed to be used in chronic conditions such as venous insufficiency and varicose veins. Moreover, a significant phlebotonic action seems to be improbable without a concomitant impact on the arterial tonus. If Fl considerably enhanced the action of norepinephrine or otherwise caused vasoconstriction, they would elevate the blood pressure [106]. Although some degree of venous tone does exist [111,112], there is no convincing evidence that the tone can be significantly influenced by Fl. On the other hand, if vasoconstriction is indeed favorable for patients with venous diseases, known vasoconstrictive agents could be used instead of Fl having unproven efficiency.

There are objective assessment methods of pharmacologic effects on the vascular bed, e.g., using isolated veins [112]. For example, dihydroquercetin did not modify the basal tone of isolated rat veins [113]. It was reported that diosmin heightens the sensitivity of SM in rat femoral veins to calcium, which might explain the phlebotonic action (the study was supported by manufacturers) [114]. On the contrary, hesperetin (the aglycone form of hesperidin) induced vasodilatation in humans and hypertensive rats [115,116]. The vasorelaxing effect was demonstrated also for eriodictyol obtained from lemon [116]. Overall, the quality of studies on this topic is regarded to be poor, favorable effects being often exaggerated [106,117]. The most rigorously conducted trial did not show any benefit from Fl in the treatment of venous leg ulcers [117]. Among positive actions, subjective improvements (life quality, pains, cramps, swelling sensation, and heavy legs) are often reported [102,109,118,119], which may be caused by a placebo effect. Admittedly, an improvement of venous hemodynamics under the influence of MPFF has been reported, confirmed by strain gauge plethysmography and ankle circumference measurements in patients with chronic venous insufficiency [101,119–122]. The data on foot volumetry have been unconvincing [103,123].

Mechanisms of supposed anti-inflammatory and anti-edematous effects of Fl are hardly comprehensible. Should anti-inflammatory or diuretic agents be indicated, it is unclear why Fl with unproven efficiency must be used instead of well-known drugs [120]. Furthermore, the anti-oxidative capacity of Fl has been discussed. Antioxidants are generally regarded to be far from the scientifically founded clinical application [1,124]. Adverse effects of Fl are reported to be mild to moderate across studies. The

most common events are skin lesions, e.g., eczema, gastrointestinal disturbances, and hypertension [96,108]. In this connection, the biological role of Fl as repellents, protecting plants from herbivores, should be mentioned. Certain Fl are toxic to insects or other organisms [100,125,126]. Presumably, Fl are mild toxins stimulating defense mechanisms [127] so that their abundant intake may be associated with adverse effects, especially in chronic disease or advanced age. Moreover, concentrations of Fl in drugs and nutritional supplements are higher than in a usual diet. Excessive amounts of polyphenols reaching the colon may cause dysbiosis [128].

Despite the above arguments, there are many papers reporting favorable effects of Fl. However, numerous studies were sponsored by the industry. Obviously, verification in large-scale independent experiments is needed. Should the useful properties of Fl be confirmed, the question will arise whether pharmaceuticals can be replaced by enhanced consumption of citrus fruit as a source of Fl. Concentrations of different Fl in citrus juices are listed in the review [99]. Remarkably, some commercial juices contain more Fl than hand-pressed ones [99] probably due to the forceful pressing and use of pulp. However, some commercial products in the former Soviet Union, labeled as citrus juices are diluted, contain added syrup, and artificial flavors.

In conclusion, the evidence in favor of the phlebotonic action of Fl is inconsistent, potential mechanisms being hardly comprehensible theoretically. The effectiveness of venoactive drugs needs verification in large-scale studies protected from conflicts of interest, using objective methods such as measurements of supramaleolar circumference, plethysmography, water volumetry, and modern optoelectronic methods.

Acetylcysteine as a mucolytic drug

This section is about acetylcysteine (N-acetyl-L-cysteine or NAC) used as a mucolytic agent hydrolyzing disulfide bonds that link together mucin monomers. Other potential fields of application such as chronic kidney disease and renoprotection, neuropsychiatric diseases, i.e., substance dependence disorders, diseases of the lungs, heart, and gastrointestinal tract are beyond the scope of this review [129,130]. A clinically significant mucolytic effect is not well documented, especially if the substance is taken per os [131,132]. There is probably a placebo effect reinforced by conditioning if NAC had been administered together with expectorants or inhalations. Analogously, the intravenous NAC was found to be ineffective in a placebo-controlled randomized study [133,134]. It was pointed out that all positive findings about NAC in chronic obstructive pulmonary diseases originated from studies either investigating small numbers of cases or conducted in groups not representative of wider populations [135]. The Bronchitis Randomized on NAC Cost-Utility Study showed that NAC is ineffective in preventing deterioration of lung functions in patients with chronic obstructive pulmonary disease (COPD) [136]. It was concluded that there had been no randomized controlled trials demonstrating benefits from inhaled NAC in the treatment of airway diseases, while no data have convincingly shown an improvement of mucus expectoration [137–140]. At the same time, there is a risk of epithelial damage when NAC is administered as aerosol [140]. Inhaled NAC was found to be ineffective in atelectasis/mucus plugging in intubated patients [141]. Aerosolized NAC was rapidly cleared from the lungs and did not alter the biophysical properties of sputum [142]. At the same time, a systematic review found that the treatment with mucolytics reduced the frequency of exacerbations in patients with COPD, whereas some studies applied NAC [143]. The latter findings were supported by a pharmaco-epidemiologic study [144], although bias was not excluded [145]. The 2013 Cochrane and other reviews concluded that there is evidence neither in favor of nebulized nor oral NAC for the routine treatment of cystic fibrosis [133,138,146]. In particular, NAC exerted no favorable effects on forced vital capacity, other important indices, and the death rate in cystic fibrosis [146].

The following considerations cast doubt on the efficiency of NAC, especially if taken per os. NAC was detected neither in airway secretions nor in bronchoalveolar lavage fluid, while cysteine concentrations did not increase in the lavage fluid following oral intake of NAC [132,133,140,147,148]. A slight increase in radioactivity of bronchial secretions after the oral intake of ^{35}S-NAC does not prove that there was a chemically active substance in the bronchial contents [149]. A controlled, double-blind study investigating oral NAC showed no significant differences in lung functions, mucociliary clearance, and sputum viscosity compared to placebo [133,150]. This is not surprising as the oral bioavailability of NAC is low (4%–10%), the substance being metabolized in the gut, liver, and other tissues, while about 30% of the clearance occurs via kidneys [133,140,151].

A separate topic is the use of NAC for the treatment of microbial infections with the formation of biofilms. The antibiotic resistance of bacteria in biofilms contributes to the chronicity of infections [152]. Biofilms were shown to be responsible for both acute and chronic inflammation of the upper respiratory tract, sinusitis, otitis media, tonsillitis, and adenoiditis [153]. Difficulties of biofilm eradication with systemic antibiotics have led to consider non-antibiotic therapies including NAC. It was reported about mucolitic efficiency of NAC against bacterial biofilms on the tonsils [130,154,155]. The evidence from *in vitro* studies indicates that NAC interferes with the biofilm formation in the nose, throat, and oral areas potentiating the action of antibiotics [156–159]. There have been *in vitro* studies reporting that NAC at relatively high concentrations lowers the sputum viscosity [160,161]. Obviously, it is easier to achieve a sufficient topical concentration in the nose, throat, and oral areas than in the bronchi.

Apart from the mucolytic action, NAC was supposed to possess antioxidative, anti-inflammatory, antimicrobial, and anticancer activity [159,162–164]. The data on the anti-inflammatory effects of NAC are limited while the mechanism is hardly comprehensible. The supposed antimicrobial activity should be tested by microbiological methods in comparison with that of antibiotics. *A priori*, suppositions about antimicrobial activity of NAC seem to be speculative. As mentioned above, synergism with antibiotics in biofilm eradication may be of clinical significance for the areas, where sufficient concentrations of NAC can be achieved. Antioxidative effects have been discussed previously [124,165]; in any case, the anti-oxidation is not directly related to the supposed mucolytic activity of NAC.

In conclusion, there are reasons to doubt a clinically significant mucolytic efficiency of NAC beyond the placebo effect, especially if the substance is taken per os. The matter can be clarified by the sputum viscosimetry using NAC concentrations comparable to those *in vivo*, by measurements of NAC concentrations in expectorated sputum from patients receiving the substance per os and by inhalations.

Stem cells and cell therapies

Lastly, a large number of publications on stem cells (SC) and cell therapies have emerged, some of them applying such terms as rejuvenation, anti-aging strategy, etc [166–168]. The size of the legal and illegal stem cell therapy in different countries is difficult to assess. "Stem cell tourism" is a growing business. Common destinations are Asia and Latin America; among other risks it should be noted that SC "tourists" are not given a proper follow-up [169]. In China, concerns have been raised regarding unsafe SC therapies, while the officialdom turns a blind eye to them. There are no liability and traceability systems and no visible penalties for non-compliance in the stem cell legal framework. In addition to the lack of safety and efficacy systems in the regulations, no specific expert authority has been established to monitor stem cell therapy [170]. Various cell therapies, including allograft, have been provided in Japan, also to patients with alternative therapeutic options and healthy individuals [171]. Some patients experienced life-threatening problems after SC treatments [172,173]. Numerous Internet sites marketing SC falsely claim that SC therapies are ready for public consumption, overpromise benefits, and downplay or ignore risks [174]. Patients often pay for SC therapies out-of-pocket [173]. It is usual practice to charge patients to be part of SC trials [169]. Admittedly, access to unvalidated therapies is sometimes in the best interest for patients [175]. The author shares the opinion that "non-standard therapy can only be provided when it is part of an appropriately constituted research" [173], free of conflicts of interest and cost less for patients. Recognizing the global nature of the stem cell market, international mechanisms for transparency and oversight are needed [170]. The industry of SC cosmetics is beyond the scope of this review. Until proven safe and effective, cosmetic procedures claiming to use SC should be viewed as experimental [176].

Topics discussed in the literature include differentiation of exogenous SC into various cell lineages, replacement of senescent, dysfunctional, and damaged cells. Remarkably, assumptions that the progeny of SC can differentiate into specialized cellular elements have not been confirmed for such a perfect SC as the fertilized ovum. In the "experiment" performed by nature—extrauterine pregnancy—no differentiation of pluripotent embryonic cells toward surrounding tissues is observed but an embryo and germinal layers are formed. The implantation of embryonic SC can result in the development of teratoma [177,178]. It is known from general pathology that a focal cell proliferation results in the formation of a nodule rather than migration of individual cells into surrounding tissues. For a pathologist, it is difficult to envisage how SC migrate in tissues such as myocardium, brain, liver, or cartilage, arrive at places where they

are supposed to be needed, and engraft in preexisting structures [179,180], commented in [181]. In osteoarthritis, SC would have to move through the dense matrix of hyaline cartilage. If even SC after an intra-articular injection are homing in superficial defects of the joint cartilage, synovial, or meniscal surfaces [182], proliferate there and produce extracellular substances, it remains unclear how the smoothness and congruence of joint surfaces is maintained, why the focal cellular proliferation does not result in excrescences crumbling into the articular cavity causing dysfunction and inflammation. Reproducible protocols to induce chondrogenesis by SC are lacking [183]. Furthermore, in publications on the therapy of liver cirrhosis, differentiation of mesenchymal and other SC to hepatocytes as well as the promotion of hepatocyte proliferation is held possible [184,185]. "The ability of mesenchymal SC to differentiate into hepatocyte-like cells makes them an ideal alternative method for treating liver fibrosis" [186]. However, potential differentiation along the same mesodermal lineage, e.g., to fibroblasts is not discussed. Such differentiation would possibly accelerate the advancement of fibrosis and cirrhosis. The theoretical basis for the cell therapy of liver cirrhosis is hardly comprehensible as hepatocytes are capable of mitosis and can hyper-regenerate building cirrhotic nodules.

SC therapies of neurodegenerative disease is a large topic; it should be mentioned briefly that doubts are persisting in regard to the ability of SC to migrate from an injection site to the target region and differentiate into required neuronal subtypes with appropriate synaptic connections [187,188]. Admittedly, many pre-clinical studies suggested that cell therapies could offer a promising therapeutic approach [187]. Two clinical trials concluded that SC transplantation did not ameliorate the symptoms of Parkinson's disease compared to the dopaminergic medications; in addition, the patients exhibited graft-induced dyskinesia. A recent commentary discussed the major shift in the goals of SC research from personalized cell therapy to a tool for mechanistic studies of human diseases [189,190]. The results of preclinical studies on SC therapy in Huntington's disease have been inconsistent [191]. In regard to Alzheimer's disease, promising results in animal models are deemed sufficient to initiate clinical trials; however, this area is notable for a poor translation to humans [189,192]. One of the main challenges is the potential immune rejection [189]. Current clinical trials are primarily stage I/II ones with safety as the main objective [193].

As for cardiopulmonary diseases, SC-based therapies are still at their preliminary stage [194]. The poor engraftment and survival of implanted cells is a challenge [195,196]. Since the early 2000s, several studies claimed that transplants of bone marrow or other SC can regenerate the rodent heart after myocardial infarction. This experience was translated to the clinic. However, efficacy in human studies has been ambiguous. It has become clear that donor cells are not forming new myocardium [197]. Alternative mechanisms have been proposed: immunomodulating, trophic, paracrine (anti-inflammatory, anti-apoptotic, anti-fibrotic, angiogenic, and mitogenic), activation of precursor cells in the microenvironment, etc [168,187,193,198–200]. It was hypothesized that SC secrete anti-aging substances [201]. However, there are no reasons to expect special functions from morphologically primitive SC or partly differentiated progenitors than from more differentiated cells. Note that the biological mission of SC is procreation rather than secretion of specific mediators. In any case, experiments with mature cells or cell-free products would be easier and less expensive. The cell-free substances mimicking paracrine effects of cell therapies can be obtained, e.g., from culture media. The latter approach would achieve a better dose standardizing than cell implantations whatever is understood by it [202].

Allogenic transplantations carry the risk of infections and immunologic adverse events [203]. Among others, this is a matter of concern when cell therapies are applied for the treatment of diseases with the participation of immune mechanisms, e.g., cardiomyopathy. In cardiology, routes of the so-called cell transplantation include transvenous, transendocardial, intracoronary, and transepicardial injections [204–207]. In this connection, sources of the cell material for intracoronary injections, e.g., abortion specimens and its purification from immunogenic components are of importance [205,208]. The infusion of autologous bone marrow cells or fractions of the patient's own blood is sometimes named autotransplantation; it is associated with lower risks than the allogeneic transplantation. However, benefits from such procedures are questionable apart from a restoration of the pool of hemopoietic cells after cytotoxic treatments that have been applied long since. Numerous cell therapies have been patented; just one recent example: cells collected from human placentas and umbilical cords were injected into acupuncture points as

a "method of treatment of ischemic angiopathy of lower extremity vessels" [209].

All said SC are a promising field of research. Studies of differentiated cells and cell-free products mimicking paracrine effects of cell-based therapies are promising as well. Cell therapies with scant evidence of efficacy should not be applied, especially in heart diseases [197]. Continuing to pour money into ineffective forms of cell therapy diverts funding from approaches that merit further investigation [197]. Many patients pay for cell therapies, but the experience is partly lost because conflicted researchers tend to overestimate positive results (if there are any) leaving adverse effects out of attention. As mentioned above, therapeutic methods with unproven effects should be tested within the framework of high-quality research shielded from the funding bias. As for animal experiments, they should also be performed by integer researchers not influenced by conflicts of interest.

Conclusion

The profligate prescribing has brought a hidden epidemic of side effects and no benefit to many patients [210]. The deception is objectionable on the grounds that it limits autonomy and breaches trust; these grounds possibly do not apply to placebos when they are prescribed under certain ethical and clinical conditions [211], although it can be problematic both on professional and ethical levels [212]. In other words, placebo therapy with misinformation of a patient can be justifiable and beneficial [213]; but it is still not a sufficient reason to publish biased information. Apparently, certain journals such as the BMJ are selecting reliable reports but others are publishing biased materials and rejecting criticism. Publication series of questionable reliability is sometimes continued without making references to published comments [2]. Speculative theories have been construed for the promotion of some drugs and dietary supplements. Publication bias with a preferable publication of positive results is a well-known phenomenon. Another tendency is that substances and treatment methods without proven effects are advertised, corresponding products patented and marketed in the guise of evidence-based medications. Several examples have been discussed in this review. Some of the topics might be not completely clarified so that arguments provided here can induce a constructive discussion. In conclusion, scientists, editors, and authorities should take measures to combat scientific misconduct and ensure the unbised character of professional publications [214].

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ORIGINAL RESEARCH

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In silico molecular docking, PASS prediction, and ADME/T analysis for finding novel COX-2 inhibitor from *Heliotropium indicum*

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ABSTRACT

Background/Aim: Inflammation is a defensive mechanism of the body that often links a number of fatal pathological circumstances. Progressive studies are in action worldwide to find drugs with better safety profile. Natural resources play a vital role in finding new drugs with lesser side effects. The current study was aimed to evaluate a commonly used medicinal plant *Heliotropium indicum* (family: Boraginaceae) to find compounds with therapeutic activity against inflammation.

Methods: The compounds of *H. indicum* were docked onto COX-2 receptor using Genetic Optimization for Ligand Docking algorithm software. In addition, Lipinski's rule, prediction of activity spectra for substances (PASS) prediction, and absorption, distribution, metabolism, and excretion (ADME) properties were analyzed using several online tools, namely, PASS and SwissADME. Post-docking analysis was performed using Discovery studio software.

Results: Molecular docking studies revealed that methyl rosmarinate (66.59 kcal/mol) has high binding affinity against COX-2 receptor followed by 2,5-Dihydroxy-3-heptadecyl -1,4-benzoquinone (58.41 kcal/mol) and Naringenin-5-methyl ether (56.43 kcal/mol). Also, it is notable that methyl rosmarinate has suitable molecular properties and binding patterns with amino acid residues in the active site of the enzyme.

Conclusion: The pharmacokinetic information and molecular docking patterns of compounds obtained in this study can pave a way in developing novel COX-2 inhibitors having anti-inflammatory potential with safer pharmacokinetic and pharmacodynamic characteristics.

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Introduction

Inflammation is a unique defense mechanism of the human body that at times is accompanied with numerous symptoms like pain, redness, swelling, and heat. Often there happens loss of function to eliminate or limit the spread of an injurious agent [1]. Infectious agents, thermal or physical injury, ischemia, and antigen-antibody interactions are the most common stimuli that mediate a series of events in the path of the inflammatory response [2].

Prostaglandins are the main mediators of inflammatory reactions that are important in the pathogenesis of several disorders like arthritis and cardiovascular disease [3]. Pain, inflammation, and hyperpyrexia are caused when arachidonic acid is converted into prostaglandins and thromboxanes by the endogenous cyclooxygenase (COX) enzyme [4–6]. This enzyme stays in two isoforms called COX-1 and COX-2. Although both the isoforms catalyze the same type of biochemical conversion, expressions of the two isoforms are different [7]. COX-1 is a constitutive enzyme, while COX-2 is an inducible enzyme. COX-1 mainly plays a role in maintaining the secretion and integrity of the

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gastric mucosa, satisfactory vascular homeostasis, and maintenance of renal function through the supply of prostaglandins. On the other hand, COX-2 is normally present in insignificant amounts and is expressed only after an inflammatory stimulus inducible by cytokines, growth factor, and other stimuli during inflammation response [8–11].

NSAIDs or nonsteroidal anti-inflammatory drugs produce their therapeutic effects through inhibition of COX, the enzyme that is responsible for the synthesis of prostaglandins. Non selective inhibition of COX iso-enzymes leads to a number of detrimental effects as they inhibit COX-1 which has some important physiological functions [12,13]. More recently, a new class of anti-inflammatory medications has been developed which is selective for COX-2. These agents spare the enzymatic activity of COX-1 at therapeutic dosages, preventing the harmful effects. Although these NSAIDs have superior benefits over non selective blockers, studies indicate that there are several side effects associated with these upon tissue contact like gastric upset, gastrointestinal irritation, ulceration, and bleeding due to the presence of a free carboxylic group in the parent drug [14,15].

The two most commonly used selective COX-2 blockers available by prescription in the United State, Bangladesh, and India are rofecoxib and celecoxib [16,17]. Although "coxib" drugs are better in efficacy, there are reports of increased risk of strokes and heart attacks with their long term use. As a result, rofecoxib and valdecoxib are no longer in the market since 2004 and 2005 respectively [18,19]. Thus, developing new agents with fewer side effects is an immense research area in the current scenario. To fulfill this objective, a great deal of tedious efforts is being given on identifying novel molecular targets that could form a way for new anti-inflammatory drugs.

The context of this current study thus emphasizes on screening out potent anti-inflammatory compounds of *Heliotropium indicum* L. family: Boraginaceae, also called as Hatishur, extensively found in low lands of Bangladesh and India. This plant is used extensively in folk medicines and has been studied for its hypotensive [20], antihyperglycemic [21], and neuroprotective effect [22]. Other evidence revealed that it showed a wide range activity against nociception, inflammation [23–25]. *In silico* molecular docking analysis was performed to find out potential anti-inflammatory activity of *H. indicum*. Docking analysis primarily predicts ligand conformation and orientation (or posing) within a targeted binding site. Analysis of chemical properties of identified compounds as drug target along with the identification of molecular features responsible for specific biological recognition is the secondary focus in this study.

Materials and Methods

Selection of ligands

A total of 21 compounds were retrieved after thorough review of the literature. Compounds, namely, Campesterol, Chalinasterol [26], Methyl rosmarinate [27], Indicine-N-oxide [28], Echinatine, Lasiocarpine [29], Estradiol [30], Indicine [31], Heliotrine [32], Lupeol [33], 7-hydroxyflavanone, Naringenin-5-methyl ether [34], Acetyllasiocarpine, Europine, Heliosupine [35], Lycopsamine [36], Europine-N-oxide, Heleurine-N-oxide [37], Rapanone [38], and Pestalamide B [39] were retrieved from the PubChem database (pubchem.ncbi.nlm.nih.gov) and the structure of 2,5-Dihydroxy-3-heptadecyl-1,4-benzoquinone [40] was sketched by BIOVIA Draw 18.1 software.

Validation of the ligands as potential therapeutic agents

The physical and molecular features of compounds play a vital role in the selection of these agents as drug candidates. The molecular properties of the listed compounds were analyzed by using SwissADME online server to validate them as potential ligands against therapeutic targets [41]. The compounds were then filtered through Lipinski's rule of five to predict their drug likeliness. Lipinski's rule of five (RO5) is a useful parameter to evaluate molecular properties of drug compounds for estimation of important pharmacokinetic parameters like ADME (absorption, distribution, metabolism, and excretion) for drug design and development [42–44]. All the compounds were suitable for further docking analysis, except Lupeol which violated Lipinski's rule for more than one parameter (Table 1). To validate them as suitable drug candidates, prediction of activity spectra for substances (PASS), an online server was used which predicts possible pharmacological effects of a compound based on its structural information. This tool compares more than 300 pharmacological effects and biochemical mechanisms of compounds and gives the probability of activity (Pa) and inactivity (Pi) values [45]. After analyzing the compounds by PASS, nine compounds were selected for further

in silico studies based on their anti-inflammatory potentials (Table 2).

Ligands preparation for docking

The finally selected nine compounds were suitable for molecular docking study. Ligands were prepared for docking using the Sybyl-X 2.1.1 Molecular Modeling Suite of Tripos, Inc. Concord 4.0 was used for generating 3D conformations of the ligands [46], and hydrogen atoms were added and charges were loaded using the Gasteiger and Marsili charge calculation method [47]. Then, the individual ligands were minimized with the Tripos Force Field applying the Powell method with an initial Simplex [48] optimization and 1,000 iterations or gradient termination at 0.01 kcal/(mol*A). Finally, the ligand files were saved at the mol2 format for docking investigations.

Protein preparation and active site determination

Crystal structure of target protein COX2 with a selective inhibitor with a resolution of 2.8 Å (PDB ID: 6COX) was collected from the RCSB protein data bank [49]. Active site of the enzyme was identified using previously given information of Kurumbail [11]. As this protein structure is dimer, only chain A was selected for the docking study. Necessary cleaning and preparations like deletion of heteroatoms, cofactor, and water were done using Sybyl-X 2.1.1. Hydrogen atoms were added to the geometry and the target protein was minimized using MMFF94s force field assigning MMFF94 charges with 1,000 steps of conjugate gradient staged minimization protocol. Target protein was saved in pdb format for docking investigations.

Molecular docking and analysis of docked poses

After preparation of selected ligands and protein structure, the docking was performed using Genetic Optimization for Ligand Docking (GOLD) software package 5.3 (Fig. 1). GOLD is a genetic algorithm-based docking protocol that explores the conformational flexibility of the ligand to the hydrogen bond interacting residues of the receptor [50]. In this protocol, the ligands are considered

Table 1. Molecular properties of the compounds by SwissADME.

Molecule	PID	MW	HBD	HBA	cLog P	MR	TPSA
7-hydroxyflavanone	1890	240.25	1	3	2.53	67.52	46.53
Acetyllasiocarpine	101915797	453.53	1	9	1.92	120.49	111.60
Campesterol	173183	400.68	1	1	6.90[a]	128.42	20.23
Chalinasterol	92113	398.66	1	1	6.80[a]	127.95	20.23
Echinatine	22384	299.36	3	6	0.32	81.14	90.23
Estradiol	5757	272.38	2	2	3.40	81.03	40.46
Europine	5462451	329.39	3	7	0.19	87.07	99.46
Europine-N-oxide	5484389	345.39	3	7	-0.88	90.43	125.65
Heleurine-N-oxide	102122200	313.39	1	5	0.74	88.07	85.19
Heliosupine	5376265	397.46	3	8	1.04	106.02	116.53
Heliotrine	906426	313.39	2	6	0.94	85.87	79.23
Indicine	73614	299.36	3	6	0.32	81.14	90.23
Indicine-N-oxide	280564	315.36	3	6	-0.40	84.50	116.42
Lasiocarpine	5281735	411.49	2	8	1.51	110.75	105.53
Lupeol	259846	426.72	1	1	7.31[a]	135.14[a]	20.23
Lycopsamine	107938	299.36	3	6	0.32	81.14	90.23
Methyl rosmarinate	6479915	374.34	4	8	2.00	95.72	133.52
DHB	-	378.55	2	4	5.85[a]	113.16	74.60
Naringenin-5-methyl ether	188424	286.28	2	5	2.02	76.04	75.99
Pestalamide B	25158705	342.35	3	5	1.64	90.71	116.33
Rapanone	100659	322.44	2	4	4.42	93.93	74.60

PID = Pubchem ID; MW = Molecular weight; g/mol (acceptable range: <500); HBD = Hydrogen bond donor (acceptable range: ≤5); HBA = Hydrogen bond acceptor (acceptable range: ≤10); cLogP = High lipophilicity (expressed as LogP, acceptable range: <5); MR = Molar refractivity (acceptable range: 40–130); TPSA = Topological polar surface area; $Å^2$; DHB = 2,5-Dihydroxy-3-heptadecyl-1,4-benzoquinone. [a] denotes violation of acceptance criteria.

Table 2. PASS data of selected compounds for anti-inflammatory activity.

Molecule	Therapeutic activity	Probability of activity (Pa)	Probability of inactivity (Pi)
7-Hydroxyflavanone	A	0.603[a]	0.031
	AI	0.334	0.048
	NSAID	0.192	0.107
Europine	AI	0.222	0.156
Heliotrine	AI	0.198	0.191
Indicine	AI	0.208	0.177
Lycopsamine	AI	0.208	0.177
Methyl rosmarinate	AI	0.534[a]	0.005
	A	0.469	0.066
	NSAID	0.167	0.138
DHB	A	0.511[a]	0.054
	AI	0.403	0.022
	NSAID	0.183	0.116
Naringenin 5-methyl ether	A	0.632[a]	0.026
	AI	0.318	0.058
	NSAID	0.179	0.120
Rapanone	A	0.511[a]	0.054
	AI	0.403	0.022
	NSAID	0.183	0.116

A = Anti-inflammatory; AI = Anti-inflammatory, intestinal; NSAID = Non-steroidal anti-inflammatory agent; DHB = 2,5-Dihydroxy-3-heptadecyl-1,4-benzoquinone. [a] denotes significant activity.

flexible while the receptor remains fixed. Docking was performed adjusting parameters of population size (100); selection pressure (1.1); number of operations (100,000); number of islands (1); niche size (2); and operator weights for migrate (0), mutate (100), and crossover (100). The binding site for the ligands was predetermined by the active site with co-crystallized ligand with a 10 Å radius sphere around it. Genetic Algorithm settings were set to default, and for each ligand, a set of 10 solutions were saved. Docking scores were evaluated by the GoldScore fitness function. This genetic algorithm scoring function is an optimized method for the calculation of binding positions of the ligand. The calculation is made using the following formula:

$$\text{Fitness} = S(\text{hb\_ext}) + 1.3750 * S(\text{vdw\_ext}) + S(\text{hb\_int}) + 1.0000 * S(\text{int})$$

$$S(\text{int}) = S(\text{vdw\_int}) + S(\text{tors})$$

where S hb_ext: protein-ligand hydrogen bond energy; Svdw_ext: Van der Waals interactions between protein and ligand; Shb_int: intramolecular hydrogen bond energy; and Svdw_ int: contribution due to intramolecular torsional strain in the ligand.

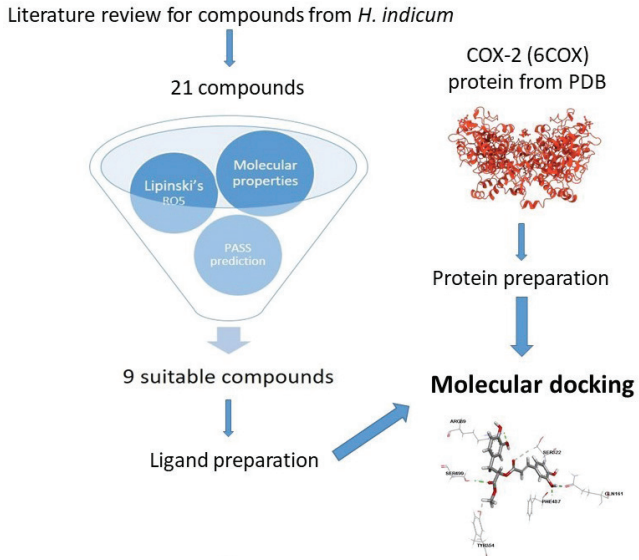

Figure 1. Graphical representation of molecular docking workflow.

Results and Discussion

Nowadays virtual screening is used extensively as it is faster and cost-effective compared to other approaches [51]. Recent advancements of computational techniques have made this as an effective alternative having positive impacts on the drug discovery process. This approach virtually screens a set of compounds and ranks them according to scoring functions. The technique involves prediction of the binding modes and binding affinities of each compound in the dataset by means of docking to an X-ray crystallographic structure [52]. In this current study, molecular docking approach of GOLD algorithm was used to evaluate docking score and interactions of the compounds with the binding site of COX-2 enzyme (PDB ID: 6COX). Molecules were screened for their molecular properties and biological activities prior to docking study to validate them as potential therapeutic targets. Goldscore fitness function was used as the parameter for binding interactions and affinity of the compounds along with a standard drug Celecoxib (Table 3). Of the total nine selected compounds, methyl rosmarinate showed highest docking score (66.59 kcal/mol) which is very close to standard drug Celecoxib (69.59 kcal/mol). Docking score of two compounds, i.e., 2,5-Dihydroxy-3-heptadecyl-1,4-benzoquinone (58.41 kcal/mol) and Naringenin 5-methyl ether (56.43 kcal/mol) also showed

promising binding affinity with the receptor (Supplementary Fig. 1b, 1g).

The best-docked compound was further analyzed for binding interactions with amino acid residues using Biovia Accelrys Discovery Studio Visualizer [53] software. This analysis showed that the methyl rosmarinate also has a similar binding mode and interactions with the COX-2 protein (Table 4). This data comply with previously reported data on synthetic compounds where amino acid residues associated with chain A of COX-2 protein were involved for protein–ligand binding interactions. Type and number of hydrogen bonds and hydrophobic interactions are a very important determinant of protein-ligand interactions as well as binding affinity [54–57]. Critical evaluation of the docking poses showed that methyl rosmarinate formed six hydrogen bonds with ARG89 (2.26 Å), GLN161 (1.65 Å), SER322 (2.72 Å), TYR354 (1.94 Å), PHE487 (2.03 Å), SER499 (2.08 Å) and with short intermolecular distances (Fig. 3a) which is suggestive that it has a good binding affinity toward COX-2. Also, the hydrogen bonding interactions were similar to selective inhibitor, celecoxib viz. ARG89, GLN161, and SER322 with corresponding bonding distance of 2.26 Å, 1.65 Å, and 2.72 Å (Fig. 2a). Of these interactions, the bond length of GLN161 was even shorter than the bond length of celecoxib (1.65 Å vs 2.31 Å), which indicates stronger binding interactions. The ligand had some hydrophobic interactions which are important for its binding with the protein. The Pi-Alkyl bonds (Fig. 3b) between methyl rosmarinate and amino acid residues of COX-2 also played a vital role in docking score. As hydrophobic interactions are one of the major driving forces of drug-receptor interactions [58], these bonds with shorter distances (<5 Å) influenced strong binding; hence, higher docking score. Hydrophobic interactions were also found identical both in Celecoxib and methyl rosmarinate (VAL318, VAL492 & ALA496) having similar bond lengths (Fig. 2b).

It is also notable that this compound has very suitable molecular properties (Table 1) and predictable pharmacological activities against COX-2

Table 3. Fitness score and protein-ligand interaction values between COX-2 and ligands.

Molecule	Gold fitness	S (hb_ext)	S (vdw_ext)	S (hb_int)	S (int)
Celecoxib	69.59	2.20	49.21	0.00	−0.28
Methyl rosmarinate	66.59	11.17	42.23	0.00	−2.64
DHB	58.41	4.23	47.55	0.00	−11.21
Naringenin 5-methyl ether	56.43	6.61	37.51	0.00	−1.76
Rapanone	55.40	4.08	42.33	0.00	−6.89
7-Hydroxyflavanone	52.62	1.05	37.51	0.00	−0.01
Indicine	45.91	2.32	34.06	0.00	−3.25
Lycopsamine	43.83	0.00	32.18	0.00	−0.43
Heliotrine	42.13	0.55	31.07	0.00	−1.14
Europine	39.52	0.13	30.97	0.00	−3.20

DHB = 2,5-Dihydroxy-3-heptadecyl-1,4-benzoquinone.

Table 4. Binding site and bond interactions analysis of the best-docked ligand along with celecoxib.

Hydrogen bonds				Hydrophobic interactions			
Celecoxib		Methyl rosmarinate		Celecoxib		Methyl rosmarinate	
Residue	Distance (Å)	Residue	Distance (Å)	Residue	Distance (Å)	Residue	Distance (Å)
ARG 89	2.20	ARG 89	2.26	VAL 318	4.49	VAL 318	4.73
GLN 161	2.31	GLN 161	1.65	TYR 354	4.97	TRP 356	4.56
LEU 321	3.03	SER 322	2.72	VAL 492	4.11	ALA 485	4.60
SER 322	2.37	TYR 354	1.94	GLY 495	4.21	VAL 492	4.21
ARG 482	2.79	PHE 487	2.03	ALA 496	4.43	ALA 496	3.75
		SER 499	2.08	ALA 496	4.29	LEU 500	4.80

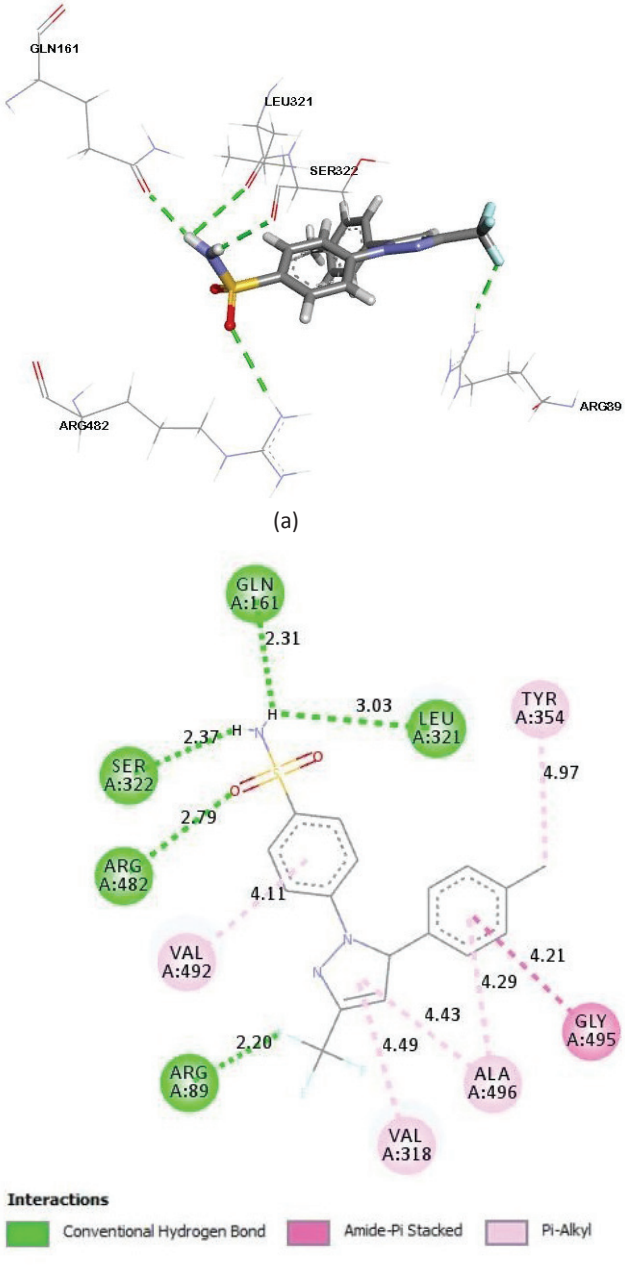

Figure 2. (a) Hydrogen bonding interactions of Celecoxib with the COX-2 protein (3D view). (b) Hydrophobic interactions and hydrogen bonds of Celecoxib with the COX-2 protein (2D view).

(Table 2). These observations further confirm that methyl rosmarinate may be an effective anti-inflammatory compound, especially with respect to

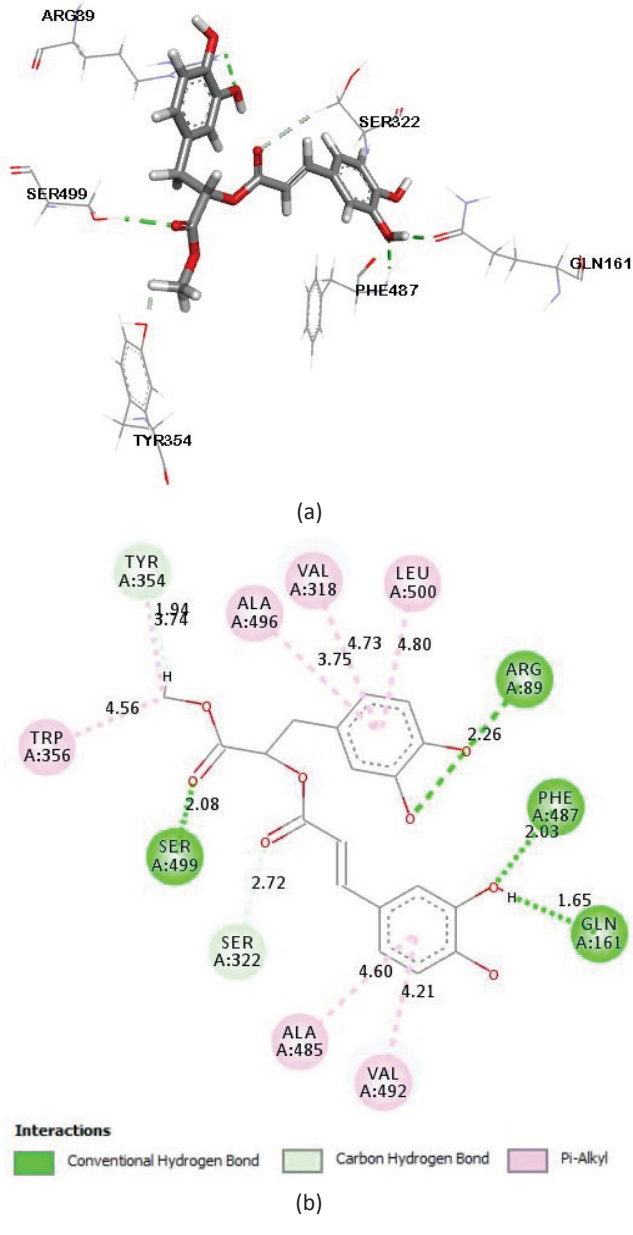

Figure 3. (a) Hydrogen bonding interactions of methyl rosmarinate with the COX-2 protein (3D view). (b) Hydrophobic interactions and hydrogen bonds of Methyl rosmarinate with the COX-2 protein (2D view).

COX-2 protein-mediated inflammation compared to other traditional NSAID drugs.

Conclusion

Development of novel compounds with therapeutic activity is an urgent need to find newer compounds

with less side effects. The present study evaluates molecular docking of phytocompounds of *H. indicum* with COX-2 protein for binding interactions. The Fitness scores of all compounds were calculated using the GOLD software. Though the binding pattern of ligands with COX-2 differed with respect to H-bonding and fitness score values, this study substantiates the hypothesis that methyl rosmarinate has the potentiality to inhibit the COX-2 protein. Hence, it is concluded that methyl rosmarinate could be a potent anti-inflammatory target molecule against COX-2 which may be worth for further clinical trials to find out its selectivity and mechanism of actions.

Conflict of interest

The authors declare that they have no conflict of interest.

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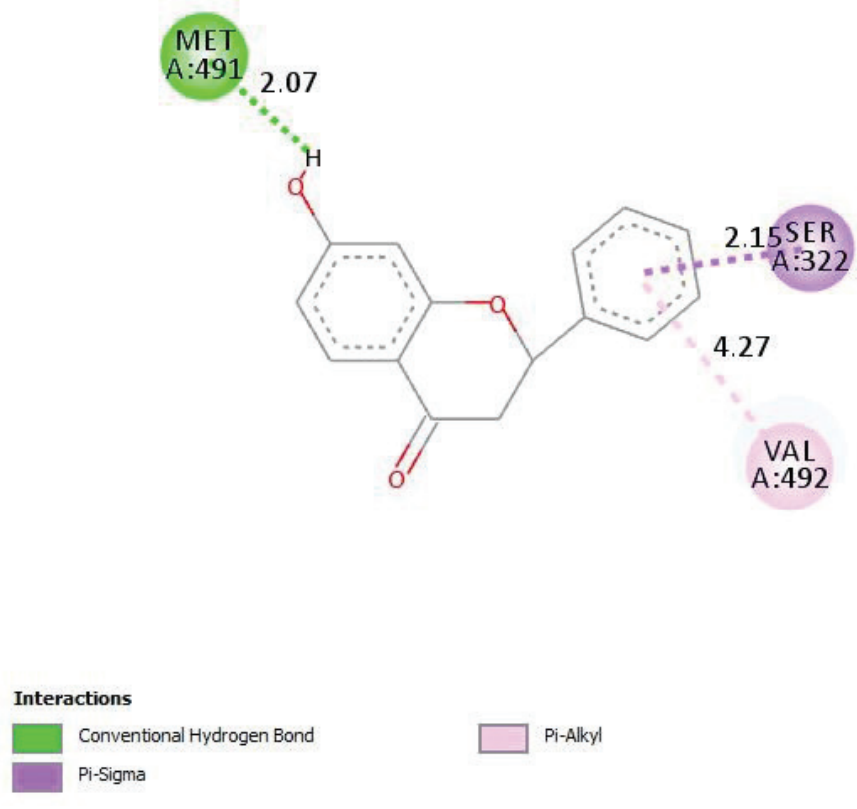
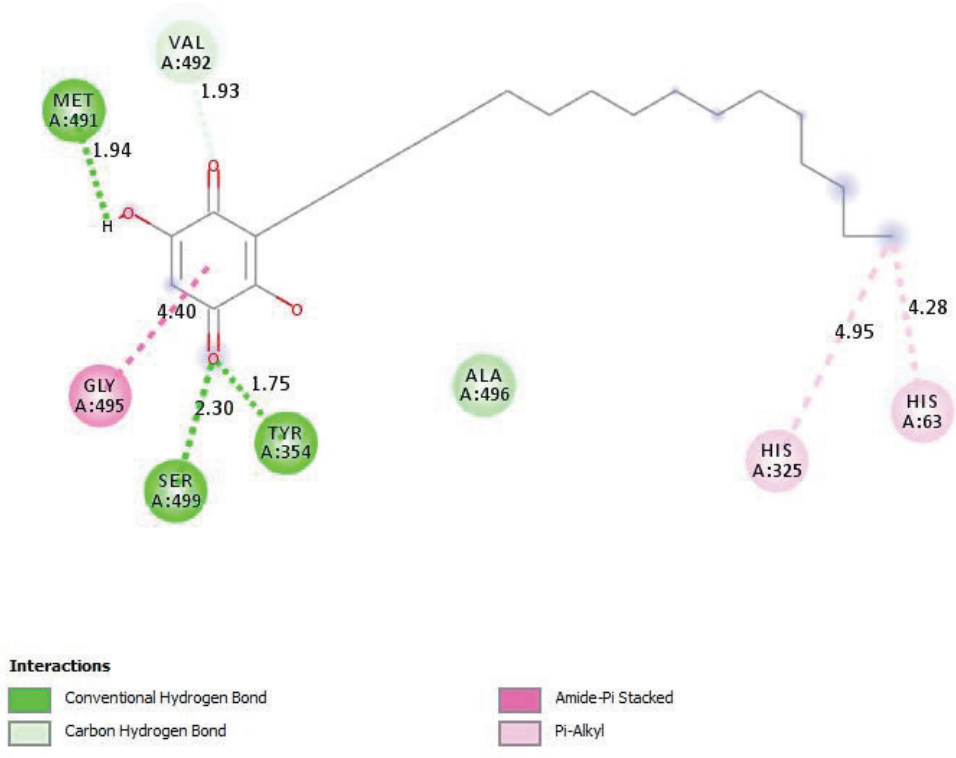
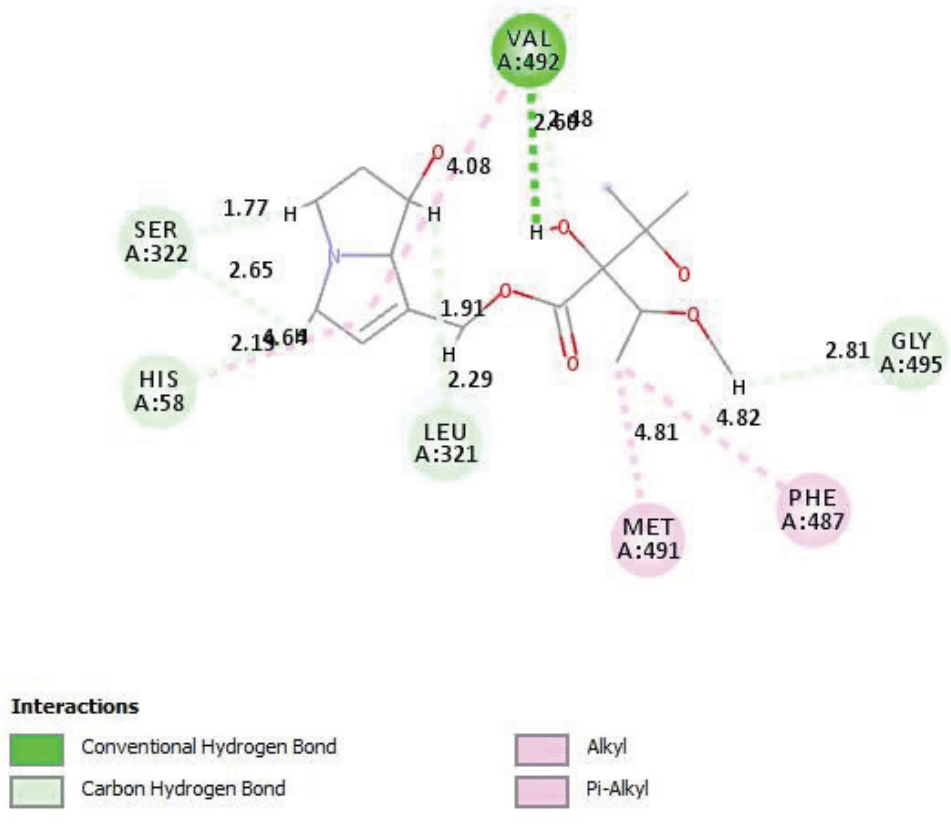
SUPPLEMENTARY MATERIAL



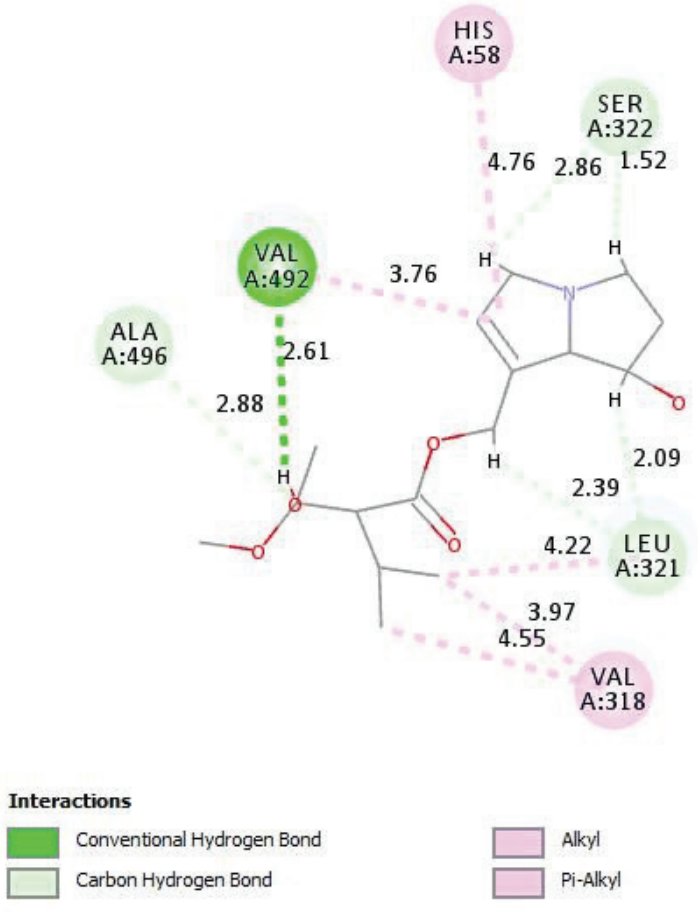
Supplementary Figure 1a. Hydrophobic interactions and hydrogen bonds of 7-Hydroxyflavanone with COX-2 protein (2D view).



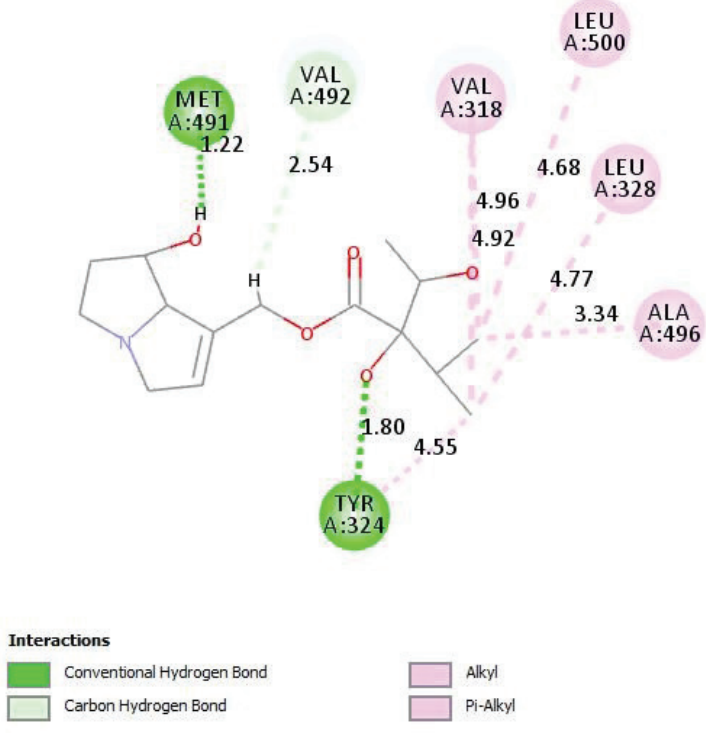
Supplementary Figure 1b. Hydrophobic interactions and hydrogen bonds of 2,5-Dihydroxy-3-heptadecyl-1,4-benzoquinone with COX-2 protein (2D view).



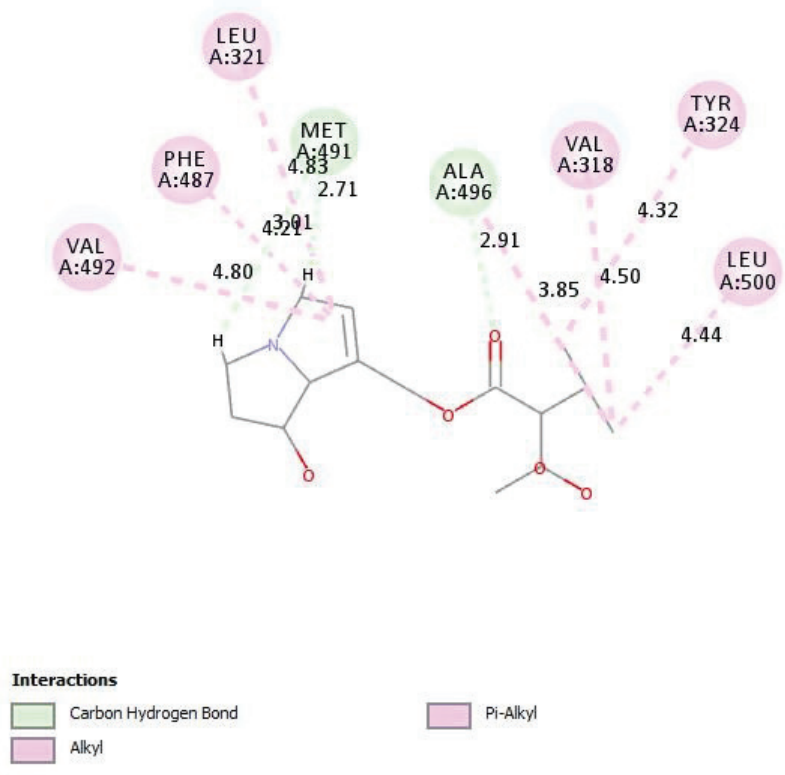
Supplementary Figure 1c. Hydrophobic interactions and hydrogen bonds of Europine with COX-2 protein (2D view).



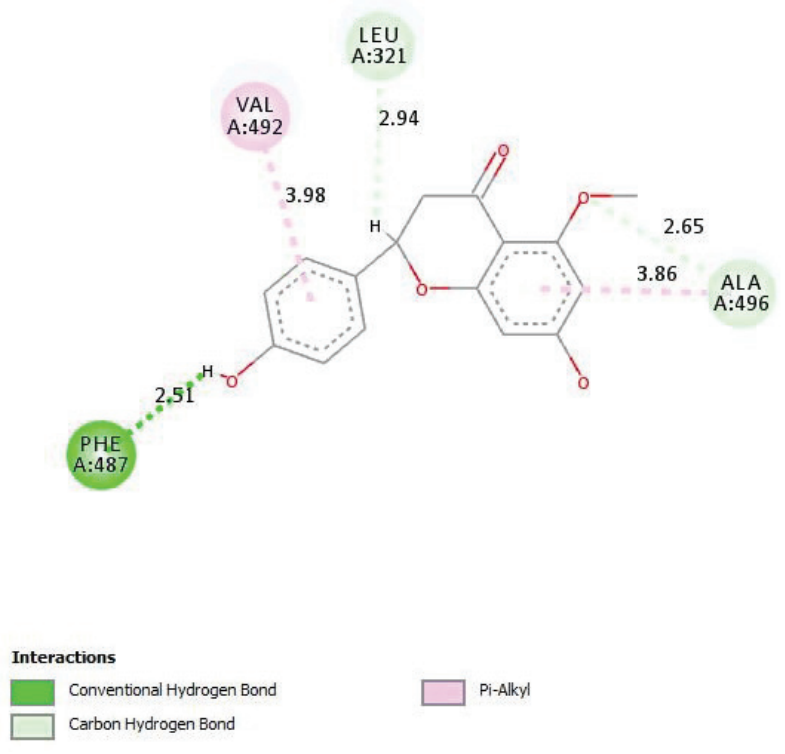
Supplementary Figure 1d. Hydrophobic interactions and hydrogen bonds of Heliotrine with COX-2 protein (2D view).



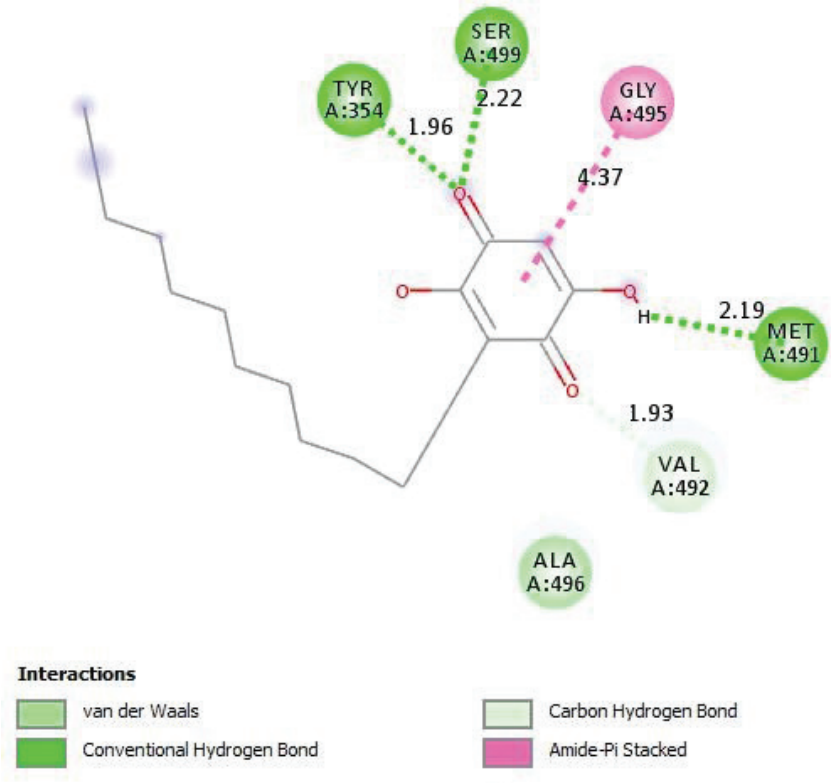
Supplementary Figure 1e. Hydrophobic interactions and hydrogen bonds of Indicine with COX-2 protein (2D view).



Supplementary Figure 1f. Hydrophobic interactions and hydrogen bonds of Lycopsamine with COX-2 protein (2D view).



Supplementary Figure 1g. Hydrophobic interactions and hydrogen bonds of Naringenin 5-methyl ether with COX-2 protein (2D view).



Supplementary Figure 1h. Hydrophobic interactions and hydrogen bonds of Rapanone with COX-2 protein (2D view).