

PHARMACOLOGICAL ASSESSMENT OF HISPIDULIN - A NATURAL BIOACTIVE FLAVONE

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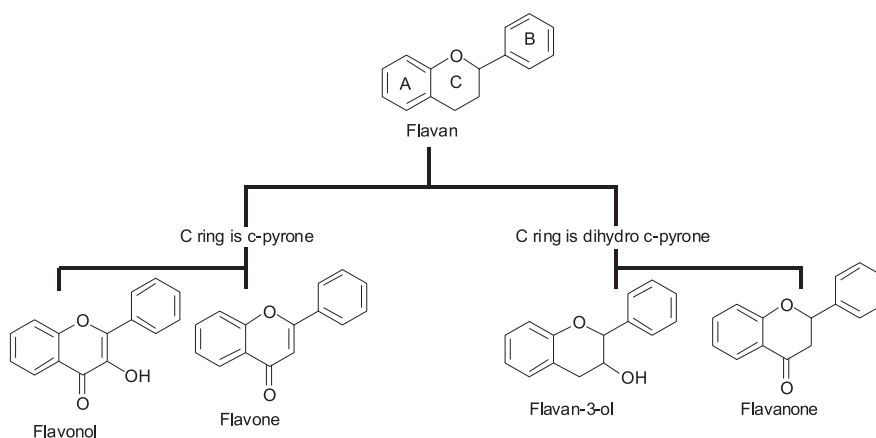
Abstract: Hispidulin is well-known natural bioactive flavone on behalf of its pharmacological aspects. This review contains data on isolation, synthetic methodology, pharmacokinetics and bioactivities of hispidulin. The article provides a critical assessment of present knowledge about hispidulin with some clear conclusions, perspectives and directions for future research in potential applications.

Keywords: antioxidant, anticancer, antiepileptic, antiinflammatory, antiosteoclastogenesis, antihypnotic, hepatoprotective, mitochondrial metabolism

Hispidulin (5,7-dihydroxy-2-(4-hydroxyphenyl)-6-methoxy-4H-chromen-4-one or 6-methoxy-5,7,4'-trihydroxyflavone or 6-methoxyapigenin or dinatin) is a polyphenolic phytochemical belonging to the flavone class of flavonoids (1). Flavones are generally crafted in herbs and cereals. Recently, flavones have grabbed scientific and public attention because of their reputed beneficial results against certain cancers, atherosclerosis and osteoporosis.

Hispidulin, a bioactive flavone, has been reported as an effective anticancer agent and the strongest ligand of benzodiazepine (BZD) site of GABA_A receptor (2-4).

Flavones are derived from flavan structure (5-7) (see Scheme 1). As displayed in Scheme 1, flavonoids contain a benzene ring (A) condensed with a six membered heterocyclic ring (C) having a phenyl substituent (B) at C-2. The saturation of ring



Scheme 1. Flavonoid classes

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C leads to the flavonol and flavone based structures, in case ring C is c-pyrone, or it might result in flavanol and flavanone if ring C is dihydro derivative of c-pyrone (8). Owing to structural diversity, flavones uncover variety of functions, which not only include biological and pharmaceutical activities (9-17), but also incorporate color control in vegetables and fruits, to protect them from UV radiations as well as infectious attacks of microorganisms (6, 18, 19).

Pharmacokinetics of hispidulin

Pharmacokinetics plays an important role in deciding about future drugs. It depends on absorption, distribution, metabolism, excretion and toxicity. For oral absorption of drugs higher polar surface area (PSA) and low molecular weight (MW) are considered good. PSA of hispidulin has been reported as 100.12 Å², which is appreciably high with MW of 300 (20). Regarding distribution of biologically active compound in human body, blood-brain-barrier (BBB), permeability and volume of distribution play vital role. An applicable amount of intact bioactive flavonoids, must reach target tissue to produce an *in vivo* effect. Permeability through BBB of chemically synthesized hispidulin in an *in situ* study has been reported comparable to highly penetrating compound - diazepam - with an uptake rate (Kin) of 1.14 mL/min/g (21). Study of absorption and metabolism of flavonoids is essential to assess their impact on human health. Research has been carried out recently on metabolism of bioactive flavonoids (22-24), which generally are absorbed through the intestine and after metabolism non-absorbed material is excreted in the bile by colonic microorganisms (25). But recently *in vitro* topical permeability of hispidulin has also been tested through pig skin model, in paste or solution form. Hispidulin has been reported for its prominent potential for topical delivery through the skin, with 0.4 mM water solubility and 0.4 nmol cm⁻² h⁻¹ predicted maximum permeation flux (26). Absorption and metabolism of hispidulin plays vital role in its biological properties, therefore, *in vivo* bioactive forms of hispidulin are important to be discussed. The *in vivo* properties of hispidulin are clearly known and it is identified as a novel natural ligand for BZD site of central human GABA_A receptor (27). For metabolic elucidation of hispidulin in large intestine, its biotransformation by the pig cecal microflora has been reported, with almost complete conversion (0.5 mM; *t_R* = 23.0 min) within 24 h of incubation. Pig cecum model has been reported suitable *ex-vivo* replacement of human large intestine (28). Hispidulin degrades into

scutellarein through *O*-demethylation. Scutellarein is an effective α -glucosidase inhibitor (29). Then, 3-(4-hydroxyphenyl)-propionic acid has been reported from scutellarein through ring opening mechanism. Another product (1,2,3,5-tetrahydroxybenzene) was theoretically expected, which transformed into acetyl-CoA and CO₂ via phloroglucinol (21). An investigation (4) has been made for any chemical modification in the structure of hispidulin while uptake by epithelial cells during intestinal absorption. A good permeation of orally administered hispidulin has been reported in its intact form through the Caco-2 cell monolayer. An absence of glucuronidated metabolites confirmed un-deteriorated passage of hispidulin through Caco-2 membrane (30).

Excretion of compound from human body depends upon its molecular weight and hydrophilicity. For hispidulin, hydrophilicity has been reported through octanol-water partition coefficient (log P) with a value of 2.479. Compounds with log P value less than 5 are reported to be sufficiently hydrophilic to reach membrane surfaces (20). Toxicological study of hispidulin indicated no tumorigenic or irritation risk, but high reproductive risk is reported. Overall drug likeness of hispidulin has been reported to be 1.11 (20).

Standard strategies for isolation and pharmacological evaluation of hispidulin

Hispidulin has attained substantial consideration for its biological and physiological prominence. It has been isolated from different parts of plants, including fresh leaves (31), dry aerial parts (32-35), flowers (36, 37), seeds (38) and roots (39, 40). Literature embraces a huge data reporting isolation of hispidulin and its derivatives from alcoholic extract of several medicinal plants (41-49). Reported data regarding isolation of hispidulin are either based upon random selection of plants (50) or follow-up experimentation (2, 51, 52). A proven critical approach has been adopted towards the isolation of hispidulin, in the compiled literature. Data reports the extraction of hispidulin from different parts of various plants in alcoholic fraction. Established screening lines have been implemented in pharmacological testing of hispidulin (2, 3, 53); models (animals, cell lines etc.) utilized in these testings were very close to final target (patient) (54-56) with parallel evaluation of cytotoxicity (2, 3) through comparison with reference compounds (2, 3, 57). Structure of hispidulin is presented in Figure 1, along with structural elucidation data.

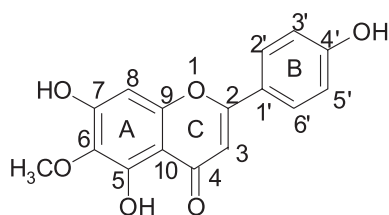


Figure 1. Hispidulin structure

$C_{16}H_{14}O_6$: 302; m.p.: 228-230°C (lit. 115).

UV: 293 (4.23), 331 (3.68); + CH_3ONa , 247 (4.30), 329 (4.44); + CH_3COONa , 294 (4.07), 330 (4.19); + $AlCl_3$, 225 (4.42), 300 sh (4.12), 316 (4.21), 394 (3.41); + $AlCl_3/HCl$, 225 (4.47), 314 (4.32), 394 (3.41) (116)

IR: 3500 (OH), 1640 (C=O γ -pyrone) (lit. 117)

MS: 302 [M]⁺, 120 C_8H_6O

CD: (c 0.001, MeOH) [θ] (nm): -10744 (300) (negative maximum), +13358 (268) (positive maximum) (lit. 115).

¹H-NMR: (DMSO- d_6): 2.80 (1H, dd, $J = 4.0$, H-3 eq), 3.10 (1H, d, $J = 12.0$, H-3 ax), 3.72 (3H, s, OCH₃), 5.42 (1H, dd, $J = 12.0$ and 4.0, H-2), 6.00 (1H, s, H-8), 6.83 (2H, d, $J = 9.0$, H-3, 5'), 7.34 (2H, d, $J = 9.0$, H-2', 6'), 12.20 (1H, s, 5-OH) (lit. 115).

¹³C NMR (DMSO- d_6): (lit. 56)

C-2	78.4	C-8	95.1	C-4'	157.9
3	42.1	9	157.6	5'	115.2
4	196.8	10	102.0	6'	127.9
5	155.0	1'	129.0	OCH ₃	59.9
6	129.0	2'	127.9		
7	159.4	3'	115.2		

Total synthesis

The literature (4) has reported total synthesis of hispidulin (Scheme 2). This synthesis was carried out with 4-benzyloxy-2,3-dimethoxy-6-hydroxyacetophenone **6** and 4-benzyloxybenzoic acid chloride **9** utilizing the Baker Venkataraman reaction (see Scheme 2c). Distinct tracks were employed for the synthesis of both compounds **6** and **9**. Synthesis of compound **6** has been reported in five steps, starting from 2,4,6-trihydroxyacetophenone **1** (see Scheme 2a). First step involved conversion of compound **1** into compound **2** through reaction with Me_2SO_4 . Second step involved selective demethylation of **2** to get compound **3** by using $AlCl_3$ /chlorobenzene. Third step involved protection of 4-hydroxy group in **3** with a benzyl group to get compound **4**. In 4th step, **4** was applied to Elbs persulfate oxidation to get compound **5**; Elbs persulfate oxidation secures synthesis of para-diphenols *via* alkaline potassium persulfate treatment of phenols. The last step gives compound **6** through methylation of hydroxyl group added in the compound during 4th step. Synthesis of compound **9** has been reported in two steps, taking 4-hydroxybenzoic acid **7** as starting material (see Scheme 2b). First step involved protection of

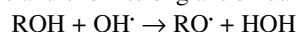
hydroxyl group with a benzyl group to obtain compound **8**. Second step synthesized compound **9** by chlorinating **8** with oxalyl chloride. Compounds **6** and **9** were reacted yielding benzoyl ester **10**, which rearranged itself into compound **11** upon KOH treatment (see Scheme 2c). Compound **11** cyclized to produce compound **12** upon thermal treatment, in the presence of acetic acid/sulfuric acid. Deprotection and selective demethylation of **12** under cold reaction conditions with BCl_3/CH_2Cl_2 resulted in hispidulin **13**.

Biological activities of hispidulin

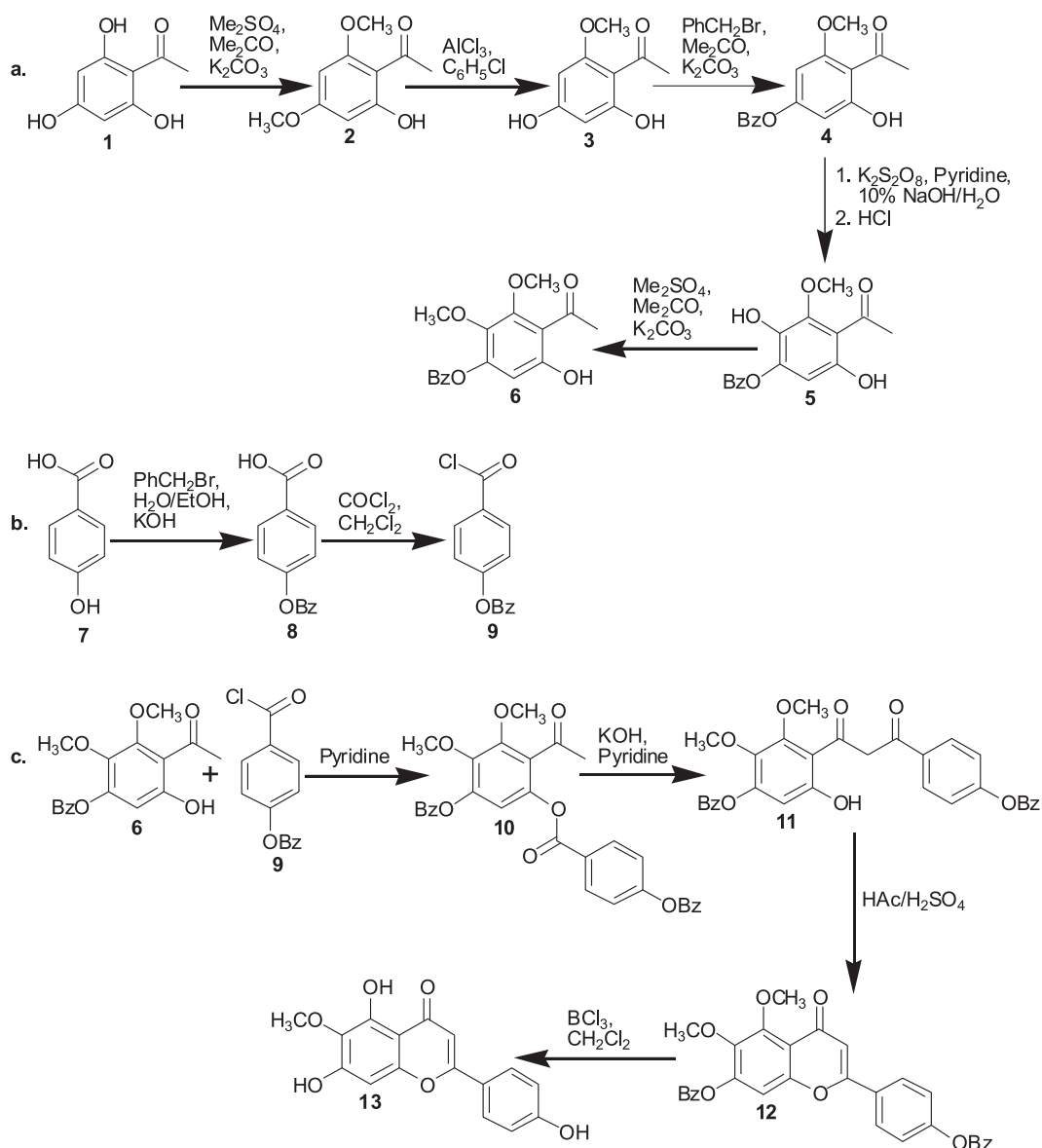
Herbal medication is evolving worldwide, but unsatisfactory certification about their safety or usefulness screens them out. This dilemma, somehow, has been resolved by the improvement and rationalization of appropriate analytical assays. For hispidulin, different pharmacological aspects have been reviewed and summarized in Table 1.

Anti-oxidant activity and effect on mitochondrial metabolism

Antioxidant character of flavonoids depends on their ability to hunt free radicals. Biomolecules can easily be spoiled by free radicals through oxidative damage (58, 59). A disproportionation among antioxidants and reactive oxygen species signifies interest of free radicals as ultimate factor, resulting in human body disorders (60, 61). Hispidulin, as an antioxidant, fights against free radicals (oxidizing agents) by making electronic dealings with biomolecules in cells (62). On the basis of fact that structure plays vital role in determining competency of antioxidants (63), two theoretically feasible reaction mechanisms have been calculated and reported from quantum data of hispidulin (1). First mechanism is related to hydrogen removal from hydroxyl groups, which mainly depends on energy required to break O-H bond i.e., bond dissociation energy (BDE). After comparing all OH groups inside hispidulin molecule, it has been reported that most stable radical for hispidulin is 4'-OH with 84.1 kcal/mol BDE energy, whereas 5-OH and 7-OH show 93.8 kcal and 88.3 kcal BDE energy (1). Molecules that require less energy to break O-H bond, breed stable free radicals and show strong antioxidant behavior.

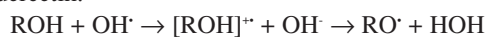


Second mechanism narrates transfer of electron from antioxidant to radical species ensuing indirect H-removal. This mechanism depends upon the energy required to craft $ROH^{\cdot+}$ radical cation, through ionization, as well as on the reactivity of radical cation. Normally, flavonoids with low IP are



Scheme 2. Total synthesis of hispidulin through Baker Venkataraman reaction using 4-benzyloxy-2,3-dimethoxy-6-hydroxyacetophenone and 4-benzyloxybenzoic acid chloride

considered as strong antioxidants (64). Hispidulin, on energy consideration, has been reported to have low IP value i.e., 6.96 eV in comparison to quercetin with IP value of 7.22 eV (1). Thus, hispidulin shows comparable antioxidant behavior to that of quercetin.



The literature (65) reports the consequences of hispidulin action on mitochondrial activity, and significance of its structure in mitochondrial respiration inhibition. Effect of this flavone on mitochondrial

metabolism has been evaluated through polarographic experiments consuming 200 μM of each flavone and mitochondrial oxidation medium. An investigation of enzymatic complexes activity in respiratory chain indicated that complex I and III provide effective reaction site (66). Hispidulin activated changes in mitochondrial behavior have been reported at various concentrations; i.e., 100, 150 and 200 μM dilutions reduce membrane electric potential and 75–200 μM hispidulin dose effects mitochondrial swelling. These results correlate mito-

Table 1. Medicinal plants rich in hispidulin.

Compound	Family	Species / Plant	Reference
Hispidulin	Asteraceae	<i>Centaurea cyanus</i>	(38)
		<i>Aegialophila pumila</i>	(41)
		<i>Centaurea pichleri</i>	(41)
		<i>Centaurea phyllocephala</i>	(41)
		<i>Centaurea malacitana</i>	(42)
		<i>Centaurea hierapolitana</i>	(47)
		<i>Centaurea phyllocephala</i>	(73)
		<i>Grindelia argentina</i>	(75)
		<i>Centaurea clementei</i>	(117)
		<i>Centaurea fufuracea</i>	(118)
		<i>Centaurea malacitana</i>	(119)
		<i>Centaurea scoparia</i>	(120)
		<i>Cheirolophus mauritanicus</i>	(121)
		<i>Centaurea chilensis</i>	(122)
		<i>Centaurea aspera</i>	(123)
		<i>Centaurea melitensis</i>	(124)
		<i>Centaurea floccosa</i>	(125)
		<i>Centaurea coronopifolia</i>	(126)
		<i>Centaurea phyllocephala</i>	(127)
		<i>Centaurea inermis and C. virgata</i>	(128)
		<i>Centaurea urvillei</i>	(129)
		<i>Centaurea aspera</i>	(130)
		<i>Centaurea arguta</i>	(131)
Lamiaceae	<i>Scutellaria repens</i>	(39)	
	<i>Scutellaria baicalensis</i>	(40)	
	<i>Salvia officinalis</i>	(43)	
	<i>Rosmarinus officinalis</i>	(43)	
	<i>Oregano (Folium origani cretici)</i>	(44)	
	<i>Sage (Folium salviae officinalis)</i>	(44)	
	<i>Thyme (Folium thymi)</i>	(44)	
Compositae	<i>Artemisia species</i>	(4)	
	<i>Salvia species</i>	(27)	
	<i>Arnica montana</i>	(46)	
	<i>Centaurea alexandrina</i>	(72)	
	<i>Artemisia vestita</i>	(110)	
	<i>Centaurea collina</i>	(132)	
Verbenaceae	<i>Lantana montevidensis</i> BRIQ.	(56)	
Hispidulin 7-O-glycoside	Plantaginaceae	<i>Plantago asiatica</i>	(45)
Hispidulin-7-neohesperidoside	Asteraceae	<i>Cirsium japonicum</i>	(48, 53)
Hispidulin-7-sulfate		<i>Centaurea bracteata</i> (aerial parts)	(49)
Hispidulin-7-sulfate		<i>Centaurea bracteata</i> (roots)	(133)

chondrial enzymatic inhibition with particular flavone structure (67). The effect of hispidulin on isolated mitochondria has also been studied (68), where 50-200 μM hispidulin has been reported to reduce mitochondrial oxygen consumption for state III, up to 42% and 27% for glutamate and succinate, respectively. Moreover, ADP to oxygen ratio decrease has also been reported (69). Along with that, hispidulin (75-200 μM) has been described to promote rate of oxygen consumption in complex IV using glutamate and succinate as the substrates. State III respiration inhibition was spotted, comparatively prominent, for succinate as substrate. Moreover, hispidulin (200 μM) effects mitochondrial respiration in the presence of glutamate to consume more oxygen in state IV (69). Electronic considerations of hispidulin structure not only style its charge distribution but also define its biological individuality. This structural speciality plays vital role in promoting its antioxidant profile. So, a better exploitation of hispidulin is anticipated in pharmacological and food related fields.

Anticancer activity

Hispidulin has the potential to control tumor progression and angiogenesis (3). In this study, mice have been induced with PANC-1 (5×10^6 each mouse) cancer cells from human pancreas, till tumor extended up to 50 mm^3 . Then, they were divided into two groups (sample mice group and reference mice group) on the basis of daily-injected dosage of 20 mg/kg , with or without hispidulin, respectively. *In vivo* analysis testified that hispidulin suppresses tumor growth of human pancreas in xenograft mice, without any toxic effect on animal's weight. *In vitro* cytotoxic analysis of hispidulin on pancreatic cancer cells and human umbilical vascular endothelial cells (HUVECs) showed receptiveness of HUVECs. This indicated prominent effect of hispidulin on angiogenesis. The IC_{50} value of hispidulin has been reported to be 20 μM in HUVECs. *Ex vivo* and *in vivo* suppression of aortic rings along with corneal neovascular growth stimulated by vascular endothelial growth factor (VEGF) has been reported. Interaction of hispidulin with distinctive molecules in HUVECs were analyzed and a suppression of VEGF-induced activation of VEGF receptor 2, PI3K, Akt, mTOR has been reported. In another study (55) *in vitro* anticancer effects of hispidulin on human esophageal, nasopharyngeal and colon cells have been reported, using Sarcoma-180 (S180) and Hepatoma-22 (H22) transplantation methods. Broad range dosage of hispidulin (2.5, 5, 10 mg/kg) was implemented for 10 days. MTT essay reported the

inhibition of tumor at 30-100 $\mu\text{g/mL}$ of hispidulin in dose dependent manner. The reported tumor inhibition rate was 25.7-67.7% (for S180) and 33.8-75.6% (for H22). The literature (54, 56) also reports *in vitro* activity of hispidulin against lymphatic, colon, breast, lung, gastric and uterus cancer in human beings. The anti-cancer effect of hispidulin on human gastric cancer cells against commercial medicines like rutin and aspirin was investigated (2). Time and concentration dependent essays testified an appreciably superior anticancer behavior of hispidulin, with IC_{50} value of 20 μM at 72 h treatment in comparison to rutin with IC_{50} value of over 500 μM . Aspirin exhibited minute inhibitory effects on gastric cancer cells (IC_{50} value calculated from graph is over 1 mM). This proves that hispidulin is 25 folds better than rutin and 50 folds better than aspirin, when it comes to their anticancer profile. In the same study, an excessive cyclooxygenase-2 (COX-2) activity in human gastric cancer cells was investigated. The sample cells have been treated with COX-2 inhibitors (celecoxib or NS-398). The observed IC_{50} of celecoxib and NS-398 after 72 h were 30 μM and 40 μM , respectively. Data prove hispidulin as an efficient anti-cancer agent, in comparison to commercial drugs.

A phytochemical study on flavonoids, including hispidulin, from *Rosmarinus officinalis* and *Salvia officinalis*, has been carried out for their anticancer activities (70). Also a constructive connection among antioxidant activity and cytotoxicity was reported (67), concluding both activities as a support either for cancer cell damage or for healthy cell protection during cancer treatment, which attributes to antioxidant exertion. Cytotoxic effect of hispidulin tested against reference anticancer drug - adriamycin, and reported values for hispidulin are less than the reference drug (71). The literature (72) reports a comparison between 30 flavonoids from different plants for their cytotoxic behavior and results indicated that methylation, particularly 6-methylation, augments cytotoxic activity of flavonoids, as in the case of hispidulin. Another research (73) reports the same observations about cytotoxic nature of methylated flavones, in the extracts of *Centaurea phyllocephala* Boiss. The data were collected either by intravenous, intraperitoneal or oral administration of the extracts in unconscious and conscious rats, respectively. The anticancer properties of hispidulin have also been reported (74) in scenario of controlled tumor necrosis factor (TNF)-related apoptosis-inducing ligand (TRAIL). In human ovarian cancer cells, hispidulin has been reported to enhance TRAIL-

induced apoptosis, moreover, it is evident from the mechanism elucidation that hispidulin activates caspases 8 and 3 that cleave poly-(ADP-ribose) polymerase (PARP), which is a key factor in programmed cell death. This organized sensitization involved adenosine monophosphate (AMP)-activated protein kinase (AMPK), which is stimulated on treatment with hispidulin. Hispidulin has also been reported (75) to exhibit most potent *in vitro* inhibitory activity against LPS-induced NO production with 98.7% at 50 µg/mL concentration. In addition, hispidulin did not show any effect on cell viability. Primarily, the ethanolic extract of *Grindelia argentina* was found to inhibit the LPS/IFN- γ -induced NO production.

Thorough “proof-of-concept” (76) about the efficacy of hispidulin in antitumor activity has been compiled from *in vitro* and *in vivo* studies, with applicable IC₅₀ values, in the presence of appropriate controls against commercially available drugs. Therefore, hispidulin is favorable contender on forthcoming anticancer drug development platform.

Antiepileptic activity

Epilepsy is a very common brain disease that disturbs about 2% of world population. Available antiepileptic drugs on the market principally work on transmitter receptors and ion channels. Roughly 30% of epileptic patients do not use these drugs, due to undesirable side effects (77). Hence, harmless and efficient antiepileptic drugs of natural product origin were strongly required, particularly to advance innovative epileptic treatments. Various scientists in this scenario had worked on hispidulin (78, 79).

Glutamate is an important neurotransmitter, in mammalian central nervous system (CNS) proficient to accelerate physiological or psychological activity. Excess of glutamate has verified association with epilepsy (80); a sudden surge of electrical activity in the brain of experimental rats has been reported upon treatment with glutamate receptor agonists (81); contrariwise, antiepileptic behavior and drop in seizure-induced brain damage has been reported in experimental animals upon treatment with glutamate receptor antagonists (82). Additionally, human epileptic patients have been reported with enhanced glutamate level (83, 84), signifying excess of glutamate as a cause of epilepsy. The reported mechanism (57) arbitrated by decline in glutamate release through exocytosis (Ca²⁺-dependent). An investigation was made to check effect of hispidulin on endogenous glutamate release to explore possible mechanisms. It was found that hispidulin constrained glutamate release

induced by K⁺ channel blocker 4-aminopyridine (4-AP) (85). Hispidulin (10 µM) has been reported to enhance γ -aminobutyric acid (GABA_A) receptor activity by 65 ± 17% (4). Stimulation of GABA_A receptor prevented voltage-dependent Ca²⁺ influx and glutamate release from nerve terminals (86). Comparative analysis of glutamate release in the presence of 4-AP (control) alone as well as 4-AP with hispidulin has been reported (57) showing a substantial reduction in glutamate release. Treatment with hispidulin (30 µM) reduced glutamate from 7.3 ± 0.1 nmol/mg to 3.6 ± 0.4 nmol/mg per 5 min; whereas 80% inhibition was observed with 100 µM concentration of hispidulin. Using dose-response relationship, IC₅₀ value of hispidulin for glutamate release inhibition has been reported as 22 µM. This signifies that a control over glutamate neurotransmission may lead to possible solution for epileptic behavior.

Neural overexcitation or scarce inhibition, generates epileptic attacks due to hyper-synchronous electrical current. Inhibition controlled by GABA_A-receptors augments in the presence of benzodiazepine (BZD) ligands. Thus, classical BZD ligands like diazepam show effective anticonvulsant activity (87). Flavonoids are reported for their effects on CNS (88). Hispidulin, being a flavonoid, has been reported (27) for antiepileptic activity through binding inhibition of flumazenil with BZD site of GABA_A receptor with IC₅₀ value of 1.3 µM. A recent study (89) has reported hispidulin as a potent ligand for BZD site of human GABA_A receptor, with 81% inhibition of maximal GABA_A response, showing strongest binding activity to BZD site comparative to ursolic acid, carnosol, oleanolic acid, salvigenin, rosmanol and cirsimaritin. Reported data about hispidulin neuropharmacology, particularly control over epileptic activity through interaction with GABA_A receptors, indicate its potential to cope with different neurological and psychiatric disorders.

Anti-hypnotic activity

Prolyl oligopeptidase (POP) enzyme contributes in numerous features of CNS function. Noteworthy increase in POP may cause depression, anxiety, anorexia, Parkinson's disease, schizophrenia and different additional neurological disorders (90). Recently, POP inhibition by different fraction isolated from *S. racemosa* Pers. has been reported (91), with IC₅₀ values from 18.2 to 30.3 µg/mL. Fractionation resulted in lupeol, oroxylin A, oroxyloside and hispidulin. The inhibitory assays at 100 µM concentration of each compound showed that

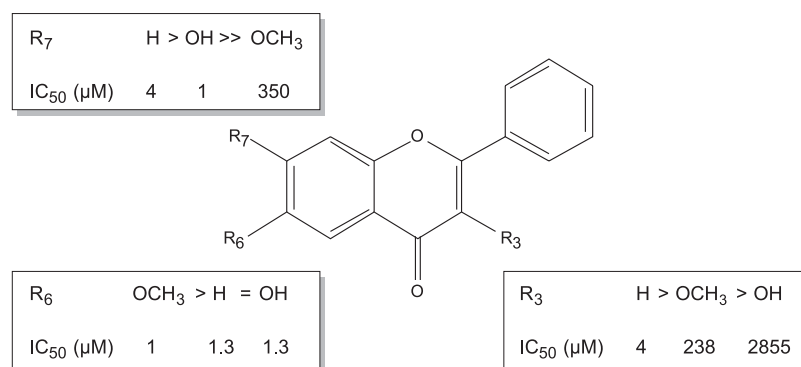


Figure 2. Effect of methoxy substitution on BZD binding affinity

hispidulin inhibited 43% of total POP activity compared to lupeol (5%), oroxylin A (20%) and oroxyloside (34%). Thus, inhibitory effects of hispidulin suggest the compound as valuable lead for a variety of brain disorders, such as schizophrenia, bipolar affect and Alzheimer's disease (92).

Sleep disorder destroys not only the cognitive function but also the immune system (93, 94). Insomnia is world spread sleep disorder, effecting chronically 10–15% of grownup population (95). Medicinal plants with sedative effect target BZD site of GABA_A receptor (87), which maintains the equilibrium in neuronal excitation and inhibition (89), to regulate sleep. Peak sedative-hypnotics are targeted through BZD binding spot of GABA_A receptor (96). Hyperpolarization of membrane, by allowing a Cl⁻ influx, induced by GABA_A is mainly initiated by BZD sites, which hangs up neurotransmission. In this way, BZD sites harvest sedative-hypnotic, anxiolytic and anticonvulsant events (97). The literature (98) has reported sedative-hypnotic activity of hispidulin. Plentiful flavonoids from terrestrial plants have been reported with their affinity for BZD site of GABA_A receptors (99). *In vitro* analysis of different substitutions particularly on flavone structure for their affinity to BZD site of GABA_A receptor has also been reported (100); an increase in binding affinity has been stated for 6-methoxylation (hispidulin), whereas 7- or 3-methoxylation resulted in significant decrease in activity (see Fig. 2). This makes hispidulin superior compared to crismaritin (7-methoxy compound) and galangin-3-methyether (3-methoxy compound) in binding affinity for BZD site of GABA_A receptor. Hispidulin has also been reported from sedative plants with binding affinity value of 8 μM (89).

Anti-osteoclastogenesis activity

Human skeleton strength depends on equilibrium between bone resorption (osteoclasts) and bone formation (osteoblasts). In osteoporosis, equilibrium shifts towards osteoclasts, and bone resorption surpasses bone formation (101), particularly in females with estrogen deficiency (102). Mechanistic pathway study revealed that osteoclast inhibition was triggered primarily by disturbance in nuclear factor κB (NF-κB), Jun N-terminal kinase (JNK) and mitogen-activated protein kinases (p38) signalling rather than extracellular signal-regulated kinases (ERK). Hispidulin tempers osteoclastogenesis and bone resorption (103). Dose dependent osteoclast inhibition has been reported with hispidulin in two different cell cultures. Hispidulin cytotoxicity was done by cell viability assay up to 10 μM without noticeable cell loss, indicating that osteoclast inhibition is not due to its cytotoxic behavior. A meaningful drop in osteoclast specific gene expression, analyzed through Reverse Transcription Polymerase Chain Reaction (RT-PCR) has also been reported for hispidulin in a concentration dependent fashion. Hispidulin is not only an effective inhibitor of bone resorption but also a remedy to control abnormal bone-lysis (104, 105).

Modern development of genetic and biological mechanistic approach in bone resorption has broadened the therapeutic concepts for antiresorptive usages. Available commercial medications may cause severe side effects like breast cancer, endometritis, thromboembolism, hypercalcemia or osteonecrosis (106, 107). Therefore, new drugs must be assessed in certain safety aspects; they should not be accumulated within bone, and have not extended existence in plasma so as to counteract the anabolic endeavor.

Table 2. Pharmacological aspects of hispidulin.

No.	Activity	IC ₅₀ (µM)	% Inhibition (at conc.)	Methodology	Reference
1	Antioxidant	NT	50 (10 ⁻⁵ M)	Lipid peroxidation inhibition	(134)
2	Anticancer	20	80* (100 µM) MTS assay (3)	MTT assay	(2)
		20 (at 72 h)	90* (100 µM)		
3	Antiepileptic	1.3	NT	H-flumazenil-BZD binding inhibition	(27)
		8	81 (100 µM)	Maximal GABA response	(89, 135)
		22	80 (100 µM)	Glutamate release inhibition	(57)
4	Anti-Hypnotic	NT	2 (10 nM)	POP inhibition	(91)
			3 (1 µM)		
			43 (100 µM)		
5	Cytotoxic	15.5 ^a (Lung Adenocarcinoma)	NT	MTT colorimetric assay	(71)
		7.6 ^a (Breast Adenocarcinoma)	NT	MTT colorimetric assay	(71)
		10.4 ^a (Cervical Adenocarcinoma)	NT	MTT colorimetric assay	(71)
		5 (Cervical Adenocarcinoma)	NT	MTT colorimetric assay	(50)
6	Anti-Influenza	NT	43 (200 µM)	NADH oxidase inhibition	(65)
		13.9 ^a	NT	Influenza H ₁ N ₁ virus neuraminidase inhibition	(52)
7	Antidiabetic	0.49	NT	DPP-IV inhibition	(136)

* Digital values estimated from graphical data presented in relevant articles. ^a µg/mL; NT = not tested; POP = Prolyl oligopeptidase.

Antiinflammatory activity

Delayed type hypersensitivity (DTH) is immune response triggering inflammatory diseases, by producing many proinflammatory cytokines. These disorders are normally treated with immunosuppressants, which have severe side effects including cytotoxicity. Hispidulin has proven antiinflammatory effects as was mentioned in various articles (3, 67, 102, 108-110).

Hepatoprotective activity

The diverse functionality of hispidulin extends to hepatoprotective effects as well, which have been reported on CCl₄ intoxicated mice (111). These effects were evaluated through concentration control of two serum enzymes, named AST (aspartate transaminase) and ALT (alanine transaminase), which highlight hepatic injury in high concentration. At a dose of 300 mg/kg of hispidulin, a decrease in AST from 70 U/L to 36 U/L and in ALT from 244 U/L to 35 U/L has been reported (111). Another study (112) has reported an association between liver injury and hepatic lipid peroxidation. Ferrandiz et al. (113) have reported positive effects of hispidulin on inhibition of hepatotoxicity induced by bromobenzene. It has been stated that bromobenzene did not affect liver weight but causes necrosis, which has been gauged by serum alanine aminotransferase (SALT) level, lipid peroxidation as malondialdehyde (MDA) equivalents and protein contents through reduced glutathione (GSH). Varied dose range (50-150 mg/kg) has been implemented to validate hepatoprotective activity of hispidulin. Reported results have shown nine times increase in SALT activity, four times increase in lipid peroxidation and five times decrease in GSH level, in toxicated animals relative to control (non-intoxicated). At a dose of 150 mg/kg of hispidulin, reported data state a decrease in SALT level from 441 to 213 (U/L), a decrease in lipid peroxidation from 271 to 104 (pmol MDA/mg protein) and a decrease in GSH from 8.4 to 5.6 (nmol/mg protein). Comparatively, non-intoxicated controls showed SALT level at 65 (U/L), lipid peroxidation at 68 (pmol MDA/mg protein) and GSH level at 42 (nmol/mg protein). Hispidulin upon comparison with reference compound - N-acetyl-L-cysteine, at the same dose, has presented promising inhibition of liver injury as well as lipid peroxidation. The promising outcomes for *in vivo* hepatoprotective pursuit of hispidulin advocates its controlled clinical studies, and indicates its candidature as future drug.

CONCLUSION

Since new drug development from natural products requires quality standards not only at isola-

tion stage but also in pharmacological screening; the data has been compiled with standard evaluation procedures, in comparison with positive and negative controls. This review article evidences imperative therapeutic effects of hispidulin for distinctive biological activities, suggesting its potential utilization in medicine, not only on cultural, anthropological and ethnobiological basis but also on pharmacological studies. After glimpsing the reported data, a logical query arises about pharmacological future of hispidulin as a potential medicine, keeping its cytotoxic consequences in mind.

Percent inhibition values (Table 2) clearly indicate that anticancer and antiepileptic activities of hispidulin are fairly higher than its cytotoxic effect even at half dosage. Moreover, it can be comprehended that cytotoxicity of hispidulin becomes insignificant at the concentrations reported for anticancer and antiepileptic activities. Hispidulin has less cytotoxic behavior and better anticancer effect than available commercial medicines. It has a proven efficacy in comparison to commercial COX-2 inhibitors. Moreover, noticeable reduction in volume and weight of cancer polyp, without affecting animal weight, signposts complimentary effect of hispidulin on normal body cells. Cytotoxic effect of hispidulin has been tested against the reference anticancer drug - adriamycin, and reported values for hispidulin are less than those of the reference drug (71).

Flavones, out of entire flavonoid group, are known to be the best for their effects on CNS, as flavones have highest binding affinity for BZD site of GABA_A receptors, and out of different flavones, hispidulin has shown maximum binding affinity (100).

DFT study has shown antioxidant capacity of hispidulin comparable to quercetin, indicating that radical scavenging nature of hispidulin has convincing competency to fight against reactive oxidizing species.

Structural characterization of hispidulin, particularly methoxy group at position-6 and hydroxyl group at position-7, upon comparative analyses against other members of the class show promising anticancer, antihypnotic and antiepileptic behavior. Methoxy group at C-6 (in hispidulin) compared to hydrogen at the same position has 760 folds higher pharmacological output. Similarly, hydroxyl group at C-7 (in hispidulin) is four times better than the hydrogen and 350 times better than methoxy group, for BZD binding affinity.

The most significant argument is that severe side effects have been reported for commercially available drugs of epilepsy and osteoclastogenesis, but hispidulin treatment reports no such side effect. Commercial antiosteoclastogenesis drugs are report-

ed to cause cancer (106, 107), while hispidulin behaves as an anticancer agent. Similarly, antiepileptic drugs show side effects like memory impairment (77), but hispidulin treatment results otherwise (114). These evidences underline the importance of research endurance on distinctive therapeutic activities of hispidulin. Petite clinical trials but convincing *in vitro*, *in vivo* and *ex vivo* literature on pharmacological aspects of hispidulin ensures that scientific platform is infantile but influential to provide technical foundation for clinical trials in any direction. It is worth mentioning that hispidulin does not show any violation to Lipinski's rule of five, as is evident from the literature reported in pharmacokinetic studies.

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