Chapter 14
Phytochemistry, Pharmacology, and Pharmacokinetics of Phytoestrogens from Red Clover Extract: An Exhaustive Overview

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14.1 Introduction

Isoflavonoids gained popularity due to their resemblance with the endogenous estrogen agonists structurally as well as pharmacologically and the potential of being used as safe alternatives in estrogen replacement therapy. They are widely distributed in the leguminous and non-leguminous families of plant kingdom (Veitch 2013). The present chapter particularly deals with the phytoestrogenic isoflavones, that is, biochanin B also known as formononetin (FMN) and biochanin A (BCA). Chemically, the BCA is written as 5,7-dihydroxy-40-methoxyisoflavone and FMN is 7-hydroxy-4′-methoxyisoflavone. The chemical structure of FMN and BCA is represented in Fig. 14.1. Although both isoflavones are extracted at high amount from the same plant, but the precursor of both FMN and BCA is different, that is, liquiritigenin (7,40-dihydroxyflavanone) for FMN and naringenin for BCA (Fig. 14.2). In the last two decades, there has been an increasing interest in these compounds as beneficial biochemical and pharmacological properties have been reported for a number of isoflavonoids. Their basic chemical structure consists of benzene ring that is linked by a heterocyclic pyran or pyrone ring and a phenyl ring (Wang 2011). The majorly distributed isoflavonoids in soy are daidzein, genistein, and glycitein and their glycoside conjugates, including 7-O-glucosides, i.e., daidzin, genistin, and glycitin (Barnes et al. 1994). In red clover, the principal

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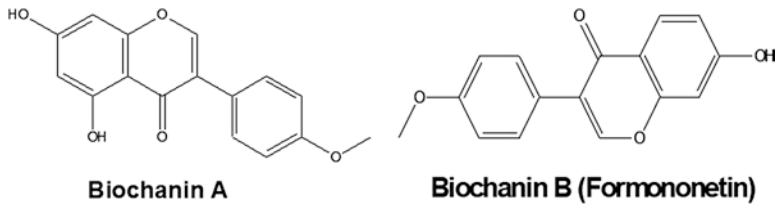

Fig. 14.1 Chemical structures of biochanins A and B

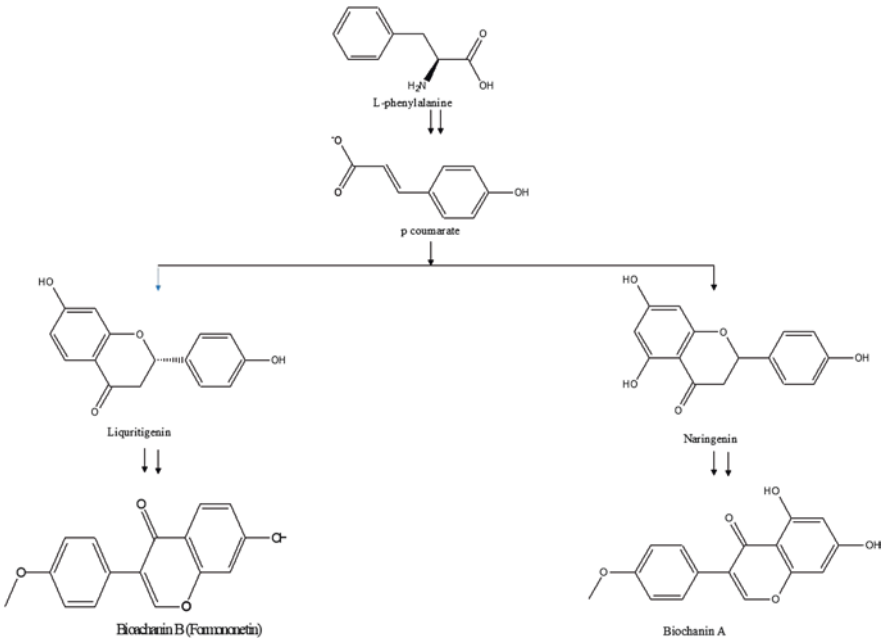

Fig. 14.2 Schematic representation of biosynthesis of BCA and FMN

isoflavonoids are FMN and BCA and their 7-O-glucosides, ononin and sissotrin (Raju et al. 2019).

Isoflavonoids are regarded as important nutraceuticals mainly due to their antioxidant effects, which give them a potential role in prevention of the various diseases associated with oxidative stress (Dixon and Pasinetti 2010). Understanding the basis of the health benefits derived from isoflavonoids requires a detailed knowledge on the absorption, distribution, metabolism, elimination (ADME), and bioavailability of these phytoestrogens. After ingestion, soy isoflavonoids are biotransformed in the intestinal tract, a process that is highly dependent on intestinal bacterial metabolism. Several major groups of colonic bacteria possess β-glucosidase activity, including *Lactobacillus* spp., *Bacteroides* spp., and *Bifidobacterium* spp. The respective isoflavone glucosides are hydrolyzed by both intestinal mucosal and bacterial β-glucosidases releasing the aglycones, which are then either absorbed

directly or further metabolized by intestinal microflora in the large intestine into other metabolites, including equol (Setchell et al. 2001; Zhang et al. 2014b). Differences in the absorption rates between the glucosylated and aglycone forms of isoflavones were reported suggesting that not all isoflavonoids can be considered in the same form if present in different types of foods (Almeida et al. 2015). The absorption of these isoflavonoids would thus seem to be controlled by enzyme specificity and distribution. After initial absorption, FMN and BCA (aglycones) undergo extensive first-pass metabolism. The resulting glucuronide and sulfate conjugates could be transported through the systemic circulation to the tissue from where they can be excreted from the kidneys or secreted into bile and return to the intestine (Spencer and Crozier 2012). In the large intestine, the microbiota further degrade isoflavones; both daidzein and genistein can be further metabolized to secondary metabolites with significant interest in the potential health effects of equol, which is a reduced metabolite of daidzein. The plasma T_{max} of daidzein and genistein metabolites typically reached 6–8 h after isoflavonoid intake (Spencer and Crozier 2012).

Cytochrome P450 enzymes (P450s or CYPs) are involved in a wide variety of biotransformations including endogenous substrates (e.g., steroids, fatty acids, prostaglandins, leukotrienes) as well as exogenous compounds (xenobiotics, as drugs, environmental toxins, food preservatives) (De Montellano 2005). P450s are primarily responsible for most of drug metabolism reactions in tissues such as the liver and the gastrointestinal tract, brain, lung, kidney, and heart (Anzenbacher and Anzenbacherová 2001). Liver microsomal P450s take part in most of the reactions involving drug biotransformations and interaction of other drugs or bioactive compounds as isoflavonoids with these enzymes may significantly affect drug action and efficacy (Zanger and Schwab 2013). There were scattered reports available in the literature on the P450 induction or inhibition, however, often with extracts and with some P450 activities only – reviewed by Taneja et al. (2015).

14.2 Pharmacological Importance of FMN and BCA

Isoflavonoids gained popularity due to their resemblance with the endogenous estrogen agonists structurally as well as pharmacologically and the potential of being used as safe alternatives in estrogen replacement therapy. They have also been found to be neuroprotective and promote neuronal survival both in vivo and in vitro. They have been tried extensively for their therapeutic activity in so many diseases, and an abundance of publications is reported in the last decade.

Isoflavones have been found to cross the blood-brain barrier due to their highly lipophilic nature. This characteristic property of FMN and BCA has been applied by many researchers for their protective activity against neurodegenerative disorders. Both have been found to improve learning, logical thinking, and planning ability. Both FMN and BCA have been suggested to be a lead molecule for treating neurodegenerative disorders such as Alzheimer's and Parkinson's. It has been shown to provide neuroprotection against cerebral ischemia through modulation of

concentration of antioxidants and inflammatory agents in the cells through Nrf2 signaling cascade (Guo et al. 2019). BCA and FMN also improve the cognitive neurobehavioral alteration through increasing the viable cells and ameliorating the degenerative cell count in cognitive deficit mice that further prove its potency towards treatment of Alzheimer's disease (Biradar et al. 2014). It has been reported to inhibit lipopolysaccharide (LPS)-induced activation of microglia and the production of TNF-α, nitric oxide, and superoxide in mesencephalic neuroglia and microglia-enriched culture (Wang et al. 2016). Furthermore, both the isoflavones had also been proved to be an antihyperglycemic molecule when tested in streptozotocin-induced diabetic rats. BCA successfully reduces the glucose and glycosylated hemoglobin levels in plasma of diabetic rats. It had also normalized the amount of plasma insulin and various enzymes involved in glucose metabolism (Harini et al. 2012). BCA also indirectly enhances the autoimmunity of host involved in protection against many fungi and bacteria by enhancing the retinoic acid receptor-related orphan receptors (ROR) α and γ that play the major role in IL-17 cascade pathway (Takahashi et al. 2017). BCA might also be a useful alternative estrogen therapy as suggested by Galal et al. for the management of renal and cutaneous changes observed in postmenopausal women (Galal et al. 2018). Despite of all the abovementioned therapeutic activity, BCA and FMN have also been proclaimed as an anticancer and anti-invasion by modulating the cell growth and migration of MDA-MB-231, MCF-7 (breast cancer cell line), and HUVEC cells (Zakłos-Szyda and Budryn 2020), hepatoprotective alone or in combination with any other hepatoprotective drug (Chaturvedi et al. 2018; Liu et al. 2016; Youssef et al. 2016) and renoprotective agent (Suliman et al. 2018) through different translational and molecular signaling cascades (Sarfraz et al. 2020).

These isoflavones possessing antioxidant activity lower the risk of certain cancers like breast cancer, prostate cancer, etc. They have also been used as expectorants in asthma (T. Li et al., 2018a) by inducing the vasorelaxation in thoracic aorta through regulating the PI3K/PTEN/Akt signaling pathway. These isoflavones have also been utilized as an alternative therapy to treat psoriasis, eczema and other skin conditions, help in reducing the blood pressure, in cardiac ischemia by inhibition of inflammasome pathway (D.-S. Wang et al., 2020) and lowering the cholesterol levels in blood. Recently, hepatoprotective activity of FMN has also been investigated against acetaminophen-induced hepatotoxicity by enhancing the Nrf2 binding (Jin et al. 2017).

Apart from innumerable human health benefits and pharmacological activity, these isoflavonoids could not be directly administered due to their low oral bioavailability resulting to less systemic exposure. This role is played by pharmacokinetics of all the compounds in which the compound is absorbed, distributed throughout the organs, and metabolized mainly by liver cytochromes (microsomal enzymes) and sometimes other enzymes present in different organs. Further, the chapter will be discussing the pharmacokinetics of both the isoflavones and their modulation by various cytochrome enzymes and membrane transporters. The multimechanistic role of BCA and FMN has been diagrammatically represented in Fig. 14.3 and Table 14.1.

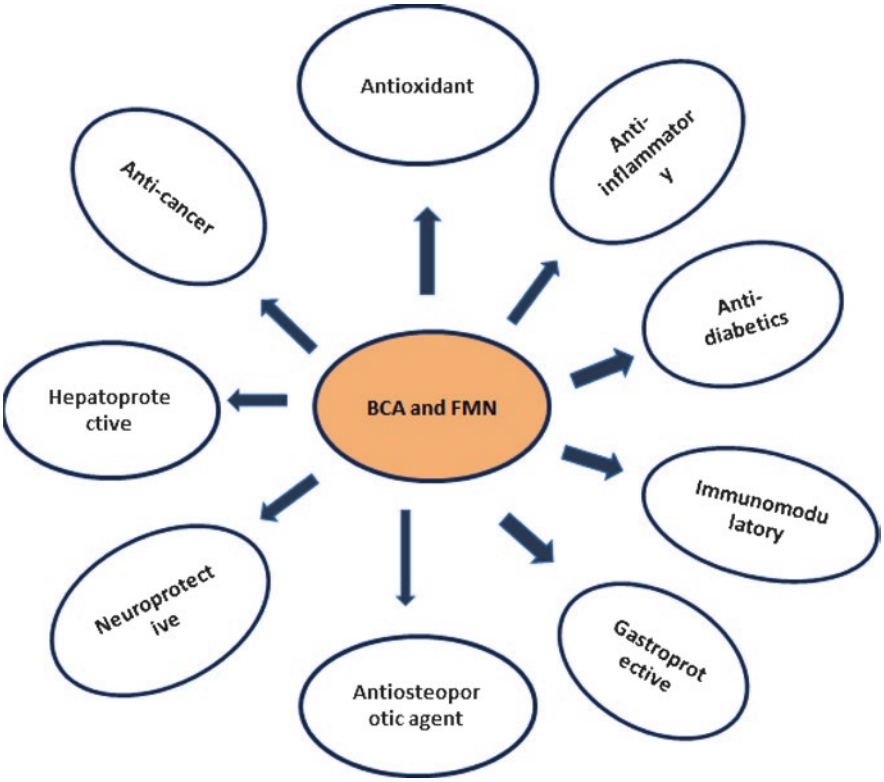

Fig. 14.3 Pharmacological activities of BCA and FMN (biochanin B)

14.3 Pharmacokinetics of FMN and BCA

The isoflavonoids exist as biologically inactive glucoconjugates in their natural form (Setchell et al. 2001). They could be only absorbed in their active aglycone form which is the result of a biotransformation reaction through an intestinal bacteria undergoing a deglycosylation pathway. They are inactive and could not be absorbed post administration in their natural form. There are literatures suggesting the conversion of inactive isoflavonoids into their active metabolite starts from the mouth itself (Allred et al. 2001). The pharmacokinetics and bioavailability information are assessed on the basis of the isoflavone's absorption, distribution, and metabolism and excretion data obtained from preclinical and clinical trials. Considering pharmacokinetics of FMN and BCA, they are reported to be metabolized into their demethoxylated isoforms, daidzein, and genistein. The schematic representation of metabolic pathway for both FMN and BCA is being detailed in Fig. 14.2. Many researchers have investigated about the pharmacokinetic characteristics of FMN and BCA under both in vitro and in vivo experimental conditions. Recently, pharmacokinetics, bioavailability, and permeation through Caco-2 cells of FMN were

Table 14.1 Various signaling pathways modulated by FMN and BCA under different pathological conditions

S. No.	Cell line\animal model	Disease associated	Signaling pathways	Dose/ concentration/ treatment exposure	Outcome	References
FMN and BCA						
1.	Male Sprague Dawley rats	Ritonavir-induced hepatotoxicity	Modulation of Nf-κB/pAkt molecules	50 mg/kg 14 days, oral	Exerts hepatoprotective effect through modulating the oxidative stress, inflammation, apoptosis, and reversing the tissue degeneration	Chaturvedi et al. (2018)
2.	ERα-positive breast cancer cells (T47D, MCF-7) Female mice (overiectomized)	Breast cancer	ERα-positive cell proliferation	2–6 μM, 48 h/2,4 mg/kg/ day, *i.p.*	Biochanin A promoted ERα-positive cell proliferation through mir-375 activation, and this mechanism is possibly involved in a mir-375 and ERα feedback loop	Chen et al. (2015)
3.	Mouse RAW 264.7 and 293T cells	Anti-inflammatory	Peroxisome proliferator-activated receptors (PPARs) modulation	10 μmol/l, 36 h	Ameliorate the cytokine secretion profile of lipopolysaccharide (LPS)-stimulated mouse RAW264.7 macrophages	Qiu et al. (2012)
4.	Male rats (Sprague Dawley)	Diabetic neuropathy	SIRT1 and NGF activator	10, 20, and 40 mg/ kg/day orally for 16 weeks	Continuous administration of FMNT-protected diabetic animals from hyperglycemia-induced neuronal damage by controlling hyperglycemia	Oza and Kulkarni (2020)
5	NSCLC cells	Non-small cell lung cancer	Suppression of EGFR-Akt-Mcl-1 axis	3 μM	Promoting ubiquitination-dependent Mcl-1 turnover might be an alternative strategy to enhance the anti-tumor efficacy of EGFR-TKI	Yu et al. (2020)

6	Male Sprague-Dawley rats, NRCMs	Myocardial ischemia/ reperfusion injury	ROS-TXNIP-NLRP3 pathway	10 mg/kg, 30 mg/kg 1 and 10 μM for 2 h	FMN notably attenuated cardiac dysfunction, infarct size, release of cardiac markers, and elevation of TNF-α, IL-1β, and IL-6. FN alleviated LPS plus nigericin-induced injury and ROS increase in NRCMs	Wang et al. (2020)
7	Saos-2 cell line-human osteosarcoma cells	Osteoporosis	Receptor activator of nuclear factor-κB ligand (RANKL)	1 μM	Regulates the expression of RANKL and OPG at the mRNA levels, as well as osteogenic differentiation markers: alkaline phosphatase (ALP), collagen type 1, and Runt-related transcription factor 2 (Runx2)	Zakłos-Szyda et al. (2020)
8	Ovariectomized (OVX) rats	Postmenopausal osteoporosis	OPG/RANKL ratio	*Cicer arietinum* extract (500 mg/kg)	Reverse TRAP5b and RANKL levels; it triggers upregulation of OPG, enhances the OPG/RANKL ratio, and modulates the bone and uterus alterations	Sayed and Elfiky (2018)
FMN						
9	HUVECs, MCF-7, BT474, and MDA-MB-231 cells	Breast cancer	Raf/MEK/ERK and PI3 K/Akt		Increase in the Akt phosphorylation Expression of Bcl-2 Proliferation induced Apoptosis inhibited	Chen et al. (2011)
10	T24 cell line	Bladder cancer	PTEN/Akt pathway	6.25–400 μM/24–72 h	Tumor growth inhibition Promoted apoptosis	Wu et al. (2017)
11	MCF-7 cell line	Bladder cancer	IGF/IGFR1/AKT pathway	30–100 μM/48 h	Inhibited the proliferation of MCF-7 cells Induced cell cycle arrest Downregulation of p-IGF-1 R, p-Akt, cyclin D1 protein expression, and cyclin D1 mRNA expression	Chen et al. (2011)

(continued)

Table 14.1 (continued)

S. No.	Cell line\animal model	Disease associated	Signaling pathways	Dose/ concentration/ treatment exposure	Outcome	References
12	ER-positive MCF-7 cells and T47D cell	Breast cancer	p38MAPK pathway	25–100 μM/48 h	Increased ratio of Bax/Bcl-2 Induced apoptosis on MCF-7 cells	Chen and Sun (2012)
13	ER-positive MCF-7 cells and T47D cell	Breast cancer	IGF-1R pathway	25–100 μM/48 h	Inhibited breast cancer growth	Chen et al. (2013)
14	MDA-MB-231 4TI	Breast cancer	PI3K/AKT pathway	2.5–40 μM/12 h	Inhibited breast cancer cell migration and invasion Reduced the expression of MMP-2 and MMP-9	Zhou et al. (2014)
15	HeLa cells	Cervical cancer	PI3K/AKT/ERK pathway	50 μM/24 h	Inhibited phosphorylation of AKT Induced apoptosis in dose-dependent manner	Jin et al. (2014)
16	HCT116 cell line	Colorectal cancer	p53 or Egr-1-related pathways	6.25–200 μM/24–72 h	Inhibit the growth of colon cancer Promoted apoptosis	Auyeung and Ko (2010)
17	SW1116 cell line HCT116 cell line	Colorectal cancer	PI3K/AKT/STAT3 pathway	20–200 μM/24 h	Tumor cells apoptosis promoted Reduced the expression of MMP-2 and MMP-9	Wang et al. (2018a)
18	RKO cell line	Colorectal cancer	ERK pathway TNF-α/NF-κB pathway	20–80 μM/48 h	Induced apoptosis Controlled cell growth	Huang et al. (2015)
19	Glioma C6 cell line	Glioma	Apoptosis signaling pathway	20–320 μM/48 h	Inhibited the growth of C6 cells in dose-dependent manner Showed synergy TMZ	Zhang et al. (2018)
20	U87MG cell line U251MG cell line T98G cell line	Glioblastoma	–	50–200 μM/48 h	Enhance the therapeutic efficacy of doxorubicin	Liu et al. (2015)

21	U266 cell line	Multiple myeloma	AKT pathway	5–60 μM/8 h	Suppress multiple myeloma	Park et al. (2018)
22	CNE1 cell line CNE2 cell line	Nasopharyngeal carcinoma	PI3K/AKT/ERK pathway	5–40 μM	Inhibited the proliferation Induced apoptosis	Qi et al. (2016)
23	A549 cell line NCI-H23 cell line	Non-small cell lung cancer	–	100–200 μM/48 h	Significantly inhibited the proliferation	Yang et al. (2014)
24	ES2 cell line OV90 cell line	Ovarian cancer	ERK1/2 pathway	20–40 μM/48 h	Significantly inhibited the proliferation	Park et al. (2018)
25	LNCaP cell line PC-3 cell line	Prostate cancer	ERK1/2 pathway	20–80 μM/24–72 h	Induced apoptosis	Ye et al. (2012)
26	PC-3 cell line DU-145 cell line	Prostate cancer	IGF/IGFR1 pathway	10–100 μM/48 h	Inhibited tumor growth	Li et al. (2014)
27	PC-3 cell line	Prostate cancer	IGF/IGFR1 pathway	25–100 μM/48 h	Induced apoptosis	Huang et al. (2014)
28	DU-145 cell line	Prostate cancer	IGF/IGFR1/AKT pathway	6.25–200 μM/48 h	Induced apoptosis	Liu et al. (2014)
29	PC-3 cell line	Prostate cancer	p38/Akt pathway	25–100 μM/48 h	Induced apoptosis	Zhang et al. (2014a)
30	MCF-7 cell-induced xenograft in Balb/c nude mice	Bladder cancer	IGF-1/IGFR-PI3K/AKT pathway	15, 30, or 60 mg/kg/day; 20 days	Decrease in the growth of tumor	Chen et al. (2011)
31	MDA-MB-231 cell-induced xenografts in Balb/c nude mice	Breast cancer	PI3K/AKT pathway and STAT3 pathway	100 mg/kg/day; 25 days;	Decrease in tumor growth Synergistic effect with sunitinib	Wu et al. (2015a)
32	MDA-MB-231-luc cell-induced xenografts in Balb/c nude mice	Breast cancer	PI3K/AKT pathway	10 or 20 mg/kg/day; 35days (once every 2 days)	Decrease in tumor growth Increase in the overall survival Decrease in lung metastasis	Zhou et al. (2014)

(continued)

Table 14.1 (continued)

S. No.	Cell line\animal model	Disease associated	Signaling pathways	Dose/ concentration/ treatment exposure	Outcome	References
33	HCT-116 cell-induced xenografts in Balb/c nu/nu mice	Colon cancer		20 mg/kg/day; 2 weeks	Decrease in the growth of tumor	Auyeung et al. (2012)
34	Human multiple myeloma U266 xenograft in Balb/c nude mice	Breast cancer	PI3K/AKT pathway	20 and 50 mg/kg/ day; 25 days	Decrease in the growth of tumor	Chen and Sun (2012)
35	PC-3 cell-induced prostate xenograft in nude mice	Prostate cancer	IGF/IGFR1 pathway	60 mg/kg/day; 20 days	Decrease in the growth of tumor	Li et al. (2014)
36	RKO tumor-bearing Balb/c nude mice	Colorectal cancer	IL6, TNF-α, NF-κB pathway	5, 10, or 20 mg/kg/ day; 14 days	Decrease in the growth of tumor	Huang et al. (2015)
37	nude BALB/c mice	Cervical cancer	PI3K/AKT/ERK pathway	20 and 40 mg/kg; 10 days	Suppressed the tumor growth	Jin et al. (2014)
BCA						
38	A549 cell line	Lung cancer	VEGF/VEGFR2 signaling pathway	100 μmol/L; 72 h	Inhibited tumor growth	Lai et al. (2020)
39	T47D, MCF-7	Breast cancer	ERα-mediated signaling pathway	24 h, 48 h	Promoted cell proliferation	Chen et al. (2015)
40	Panc1 and AsPC-1	Pancreatic cancer	Akt and MAPK signaling pathways	40–100 μmol/L; 72 h	Inhibited cell growth and exhibited cell survival	Bhardwaj et al. (2014)
41	A549 and 95D cells	Lung cancer	Bcl-2 and caspase-3 pathways	50, 100, 200, and 400 μmol/L; 24, 48, and 72 h	Inhibited cell proliferation Induces cell cycle arrest and induced apoptosis	Li et al. (2018b)
42	LNCaP cells and DU145 cells	Prostate cancer	Intrinsic (receptor-mediated) and extrinsic pathways	20–100 μM; 48 h	Induced apoptosis and inhibited tumor growth	Szliszka et al. (2013)

43	SK-Mel-28 melanoma cell	Malignant melanoma	NF-κB and MAPK signaling pathways	0–100 μM; 48 h and 72 h	Inhibited cell migration and invasion Induced apoptosis	Xiao et al. (2017)
44	A427:AML-193 co-culture cells	Lung cancer	–	5, 20, and 40 μM; 24h	Induced epithelial mesenchymal transition	Wang et al. (2018b)
45	HepG2, Huh-7 cells, and HCC cell lines	Liver cancer	Mitochondrial apoptosis signaling pathway	0.5–50 μM; 72h	Enhanced anti-proliferative Enhanced pro-apoptotic Inhibited cell growth	Youssef et al. (2016)
46	F98, U87, U251, and GL261 cells	Brain tumor	–	10, 50, 100 μmol/L; 4 days	Exhibited glioma cellular toxicity	Sehm et al. (2014)
47	FaDu cells	Head and neck cancer	P38, MAPK, NF-κB, and Akt signaling pathways	25, 50, and 100 μM; 24 h and 48 h	Reduced apoptosis Inhibited wound healing migration and proliferation of cells	Cho et al. (2017)
48	MG63 and U2OS cell line	Osteosarcoma	Mitochondrial pathway	0–80 μM; 48 h	Enhanced apoptotic rates Reduced migration and invasion ability	Zhao et al. (2018)
49	U-87 MG and T98 G	Glioblastoma	PI3K/AKT, Wnt, and Nf-κB signaling pathway	70 μM; 72 h	Enhanced inhibitory effects in combination with temozolomide	Desai et al. (2019)
50	MG63	Osteosarcoma	TP53 pathway	2–32 μM; 6 h, 12 h, 24 h, and 48 h	Suppressed the proliferation	Luo et al. (2019)
51	C6 and bEnd.3	Malignant gliomas	ERK/AKT/mTOR	5–100 μmol/L; 72 h	Inhibited factor-1α Inhibited vascular endothelial growth factor	Jain et al. (2015)
52	MG-63 and U2OS	Osteosarcoma	Intrinsic pathway	5, 10, 20, or 30 μg/mL; 24 h	Induced cell apoptosis and cell death	Hsu et al. (2018)
53	A549 cells	Anti-H5N1 influenza	AKT, ERK 1/2, and NF-κB pathway	40 μM; 48 h	Suppressed formation of ROS	Michaelis et al. (2014)
54	RBL-2H3 cells	IgE-mediated allergy	Akt and MAPK pathways	12.5, 25, and 50 μM; 2 h	Inhibited production of intracellular ROS Promoted phosphorylation	Chung et al. (2013)
55	BV2 microglial cells	Brain inflammation	MAPK pathway	1.25, 2.5, 5 μM; 36 h	Inhibited the LPS-induced phosphorylation	Wu et al. (2015b)

studied after oral and IV administration to rats at a dose of 20 mg/kg and 4 mg/kg. The investigators found that FMN showed absolute bioavailability of 21.8% after oral exposure with sufficient systemic exposure. Upon scrutinizing through Caco-2 cells, the efflux ratio of FMN was found to be within the range of 1.0–1.5, suggesting that there was no significant difference between permeability in either direction of bilayer, and transportation is basically across intestinal epithelial cells and was mainly through passive diffusion (Luo et al. 2018). The results from the latter mentioned research also manifest that FMN is largely absorbed from the large intestine of the gastrointestinal segment followed by the small intestine. This indicates the site-specific distribution of FMN and other related isoflavonoids (Luo et al. 2018). Similar pattern was also followed by BCA in another experiment performed by Jia and colleagues (Jia et al. 2004). The same was also justified by earlier studies through single-pass intestinal perfusion model (Liu et al. 2013). Zhang et al. have determined the pharmacokinetic behavior of FMN along with other isoflavones after oral administration of Buyang Huanwu Decoction to rats using tandem mass spectrometric technique (Zhang et al. 2011). Furthermore, upon oral administration of a cardio-cerebral vascular protective Chinese traditional medicine, Buyang Huanwu Tang, FMN was found to be at the second largest isoflavonoids which have the highest area under the curve concentration (Jia et al. 2004). A recent report has suggested the single pass intestinal perfusion technique to be the applicable agent for physiological based pharmacokinetic model to predict assess the human absorption of these aglycones (Liu et al. 2019).

Furthermore, FMN and BCA are also known to be metabolized by some demethylating enzymes of *Eubacterium limosum* (Hur and Rafii 2000). The bioconversion of FMN and BCA by glucosyl and malonyltransferase enzymes produces the most important bioactive isoflavones known as daidzein and genistein (Nielsen et al. 2000). Both FMN and BCA are extensively and majorly known to be metabolized in liver undergoing the glucuronidation and sulfation reaction. Moreover, interindividual and genetic variability due to ethnic and seasonal differences in food phytoestrogen are also the paramount factors to affect the metabolism of these isoflavonoids (Křížová et al. 2019). Further, the metabolite daidzein gets converted to dihydrodaidzein followed by major metabolism in to desmethylangolensin and minorly to equol (reported in one-third of the human population). Moreover the bioactive molecule genistein also gets further metabolized in to dihydrogenistein followed by formation of p-ethylphenol. Microbial culture studies gave evidence of conversion of genistein into 5-hydroxyequol, but there is no investigation performed regarding this conversion at clinical level (Matthies et al. 2012). Enzymes like lactase-phlorizin hydrolase, enterocytic/microbial β-glucosidase also take part in the intestinal gut wall metabolism of these phytoestrogens. Despite the contribution of intestinal enzymes, their enzymatic conversion to their active metabolite also took place through hepatic phase I and phase II microsomal enzymes. The major pathway of metabolism is the demethylation at 4′methoxy group followed by hydroxylation by liver enzymes. The extent of metabolism through gut microsomes is far lesser than the liver microsomes. The rat liver microsomes could metabolize the BCA to genistein more rapidly as compared to the human liver microsomes (Křížová

et al. 2019). Metabolism of isoflavonoids including BCA and FMN by phase II enzymes (uridine 5′-diphospho-glucuronosyl transferase and sulfotransferase) has also been reported instead of lower glucuronidation rate after oral administration of red clover extract. In some leguminous plants such as *Medicago truncatula*, FMN is known to get metabolized to a phytoalexin medicarpin, a pathogenic-resistant compound (Liu et al. 2017) and recently found to be an osteoprotective agent (Taneja et al. 2020). In vitro metabolic conversion of metabolite daidzein to 6-hydroxy daidzein by catechol-O-methyltransferase enzymes has been explored by one of the researchers. Same as other isoflavonoids, they are also reabsorbed again and undergo the enterohepatic circulation. Much like any other endogenous substance or xenobiotic, enterohepatic circulation and biliary elimination complicate the pharmacokinetic profiles of the majority of isoflavonoids. Extensive first-pass metabolism and biliary excretion are thought to be the causative agents for their lower bioavailability which is a major bottleneck in advancement of isoflavonoids as therapeutic agents. The metabolism of the isoflavones seems to be complicated and has not been studied extensively. However, the significant metabolites reported are daidzein and genistein in most of the cases. The newer metabolites need to be identified and evaluated for their biological activity and therapeutic efficacy if any.

These FMN and BCA are also present in the daily dietary intake of animals in high concentration. In earlier studies, the data indicates that unlike humans, these isoflavonoids are directly metabolized in to equol (Blair et al. 2003) followed by conversion to an inactive p-ethylphenol (Wocławek-Potocka et al. 2013). When investigated for the metabolism of FMN in sheeps, it is firstly demethylated to daidzein followed by further formation of equol through hydrogenation. The in vitro studies that described the incubation of FMN and BCA in the bovine rumen fluid showed that the half-lives were 4.3 h for FMN and 3.9 h for BCA (Křížová et al. 2019). The concentration of BCA and FMN was found to be only 0–9% of ingested BCA and 7–16% of FMN in the omasum of dairy cows pattern similar to sheep (Njåstad et al. 2014).

The lower and limited bioavailability of isoflavonoids could also be due to the extensive first-pass metabolism of isoflavonoids and their rapid elimination from the body. The maximum time to reach the maximum plasma concentration is within 4–8 h. In a study, the systemic levels of BCA after dietary intake were found to be minimal as compared to the administration of prepared herbal formulation. However, in some studies, it has been seen that exposure of BCA as a pure compound shows a level of saturation during absorption. The bioavailability of BCA when administered orally at a dose of 5 mg/kg is 2.6% and 1.2% at 50 mg/kg, suggesting the rapid absorption, extensive first-pass metabolism, and biliary elimination by the authors for its low bioavailability (Moon et al. 2006). There are also investigations revealing the higher bioavailability of BCA that could be the result of competitive inhibition and modulation of metabolic enzymes and transporters due to some other structurally similar compounds. This low bioavailability could be enhanced by administration of mixture of isoflavonoids that would also aid in increasing the therapeutic efficacy of isoflavonoids. However, there are reports that refuse the fact of accumulation of isoflavonoids upon long-term administration. The apparent isoflavone

bioavailability in healthy children is found to be 30–40% higher relative to healthy adults. The differences in gender do not affect the bioavailability of isoflavonoids to much higher extent. The mode of excretion of these isoflavonoids is through urine and bile and sometimes through feces. The excretion of these isoflavonoids by urine is determined by the type of the dietary product consumed and its composition regarding isoflavones and also on the nature of the isoflavone. Foods rich in isoflavone glucosides have poor urinary isoflavone excretion. However, a few studies report that the rates of urinary isoflavone excretion are not affected significantly by the nature of the food. Daidzein excretion was found to be markedly lower in equol producers as compared to equol non-producers. The primary metabolites found are glucuronide conjugates (70–90% of the total urinary isoflavones), sulfate conjugates (10–25%), and aglycone forms (1–10%). A multiethnic population study reported that Japanese, Chinese, or Native Hawaiian ancestry women excreted more isoflavones in urine than Caucasian and Filipino women. Fecal elimination as mentioned above is a minor route of elimination. Only up to 4% of the dietary isoflavones are eliminated by this route. Aglycone of the isoflavones forms the principal (>80%) part of the fecal isoflavone content.

14.4 Interaction with Cytochrome P450 Enzymes

Cytochrome P450 enzymes are the metabolic enzymes constituting the hemeprotein involved in the enzymatic conversion of many drugs, steroids, carcinogens, xenobiotics, hormones, fat-soluble vitamins, and other chemicals into their inactive and active forms. These enzymes have been the responsible facet for number of reactions inside the body which indirectly implicates their role in drug-drug interaction, drug-herb interaction, side effects, adverse effects, and unwanted increase and decrease in plasma concentration of therapeutically active drugs that lead to their toxicity and less response towards the targeted disease. Furthermore, the herbal drugs are widely used throughout the world along with the prescribed allopathic medicines; hence, the maximum possibility of herb drug interactions occurs at this stage. Various IC50 values of both the isoflavonoids are mentioned in Tables 14.2 and 14.3. Moreover, FMN and BCA are among the extensively administered isoflavonoids owing to their therapeutic beneficial properties. Taking into consideration, their pharmacokinetic and pharmacodynamic interactions with other drugs are summarized in Table 14.4.

Zapletalova and colleagues have unveiled the isoflavonoids safety efficacy by systematically investigating the interaction potential of 12 isoflavonoids with nine isoforms of cytochromes (Kopečná-Zapletalová et al. 2017). He found the genistein and daidzein and the metabolites of BCA and FMN to be the most potent inhibitors of cytochrome enzymes that non-competitively inhibit the CYP2C9 and CYP3A4. CYP3A4 was also efficiently inhibited by the parent isoflavonoid BCA, but FMN

Table 14.2 In vitro and in vivo cytochrome enzymes inhibition profiling due to exposure of BCA

S. No.	Enzyme subtype	Test compound	Model	IC50 value	References
1	1A2	7-ethoxyresorufin O-deethylation	Cryopreserved human liver microsomes	>100 μmol/l, 38.57 μM	Arora et al. (2015), Kopečná-Zapletalová et al. (2017)
		Phenacetin O-deethylation	Human liver microsomes	24.98 μM	Arora et al. (2015)
			Rat liver microsomes	11.86 μM	Arora et al. (2015)
2	2A6	Coumarin 7-hydroxylation	Cryopreserved human liver microsomes	>100 μmol/l	Kopečná-Zapletalová et al. (2017)
3	3A4/3A2	Testosterone 6b-hydroxylation	Cryopreserved human liver microsomes	65.11 ± 3.97 μmol/l	Kopečná-Zapletalová et al. (2017)
		Midazolam 10-hydroxylation	Cryopreserved human liver microsomes	>100 μmol/l	Kopečná-Zapletalová et al. (2017)
		Nifedipine 4′-hydroxylation	Human liver microsomes	>100 μM	Arora et al. (2015)
			Rat liver microsomes	51.05 μM	Arora et al. (2015)
4	2B6	7-ethoxy-4-(trifluoromethyl) coumarin 7-deethylation	Cryopreserved human liver microsomes	>100 μmol/l	Kopečná-Zapletalová et al. (2017)
5	2C8	Paclitaxel 6-hydroxylation	Cryopreserved human liver microsomes	88.25 ± 5.46 μmol/l	Kopečná-Zapletalová et al. (2017)
6	2C9/2C11	Diclofenac 40-hydroxylation	Cryopreserved human liver microsomes	>100 μmol/l	Kopečná-Zapletalová et al. (2017)
		Diclofenac 4-hydroxylation	Human liver microsomes	40.13 μM	Arora et al. (2015)
			Rat liver microsomes	>100 μM	Arora et al. (2015)
7	2C19	S-mephenytoin 40-hydroxylation	Cryopreserved human liver microsomes	>100 μmol/l	Kopečná-Zapletalová et al. (2017)

(continued)

Table 14.2 (continued)

S. No.	Enzyme subtype	Test compound	Model	IC50 value	References
8	2D6/2D4	Bufuralol 10-hydroxylation	Cryopreserved human liver microsomes	>100 μmol/l	Kopečná-Zapletalová et al. (2017)
		Dextromethorphan O-demethylation	Human liver microsomes	>100 μM	Arora et al. (2015)
			Rat liver microsomes	>100 μM	Arora et al. (2015)
9	2E1	Chlorzoxazone 6-hydroxylation	Cryopreserved human liver microsomes	>100 μmol/l, 57.56 μM	Arora et al. (2015), Kopečná-Zapletalová et al. (2017)
			Rat liver microsomes	>100 μM	Arora et al. (2015)

inhibited the same only about 20–30% of the total concentration. Hence, he concluded the maximal possible pharmacokinetic interaction of other drugs with these isoflavonoids. Moreover, Arora et al. have predicted the in vivo potential of CYP metabolic interaction of FMN and BCA using human and rat liver microsomal data obtained from in vitro studies (Arora et al. 2015). In the aforementioned investigation, they concluded that both FMN and BCA showed concentration-dependent competitive inhibition of CYP1A2 activity in human and rat liver microsomes, respectively. CYP2D6 inhibition was also perceived by FMN as concluded by the researchers. The in vivo prediction data showed the significant level of inhibition of both the isoflavonoids at intestinal level but non-significant at the hepatic level. Thereby, they have suggested for the special care to be considered during the administration of these isoflavonoids along with any prescribed drug which is metabolized by the enzyme CYP1A2. In an another study, researchers have evinced the harmful effect of red clover extract containing FMN and BCA administered to breast cancer patients (Dunlap et al. 2017). They investigated the red clover effect on metabolic CYP enzymes using the non-malignant ER-negative breast epithelial cells (MCF-10A) and malignant ER-positive breast epithelial cancer cell line, and the quantification of methoxy estrogen metabolites was performed using LC-MS/MS technique. They found that there was no effect of red clover in MCF-10A cells, while the expression of CYP1A1 was downregulated in MCF-7 cell line. These data suggest that the isoflavonoid containing red clover extract has distinctive effect on both the cells. Therefore, it is necessary to avoid red clover extract and formulations composed of these isoflavonoids to the breast cancer patients.

In addition to the pharmacokinetic assessment, these CYP450 enzymes are also being targeted by isoflavonoids for therapeutic benefits. Taking an example, FMN has been supposed to be mechanistically involved in suppression of colorectal cancer by modulation of CYP 1A1 isoform of CYP450 (Zhang et al. 2019). Furthermore, BCA has also been reported to follow similar pattern as it is found to

Table 14.3 In vitro and in vivo cytochrome enzymes inhibition profiling due to exposure of FMN

S. No.	Enzyme subtype	Test compound	Model	IC50 value	References
1	1A2	7-ethoxyresorufin O-deethylation	Cryopreserved human liver microsomes	>100 μmol/l	Kopečná-Zapletalová et al. (2017)
		Phenacetin O-deethylation	Human liver microsomes	13.42 μM	Arora et al. (2015)
			Rat liver microsomes	38.57 μM	Arora et al. (2015)
2	2A6	Coumarin 7-hydroxylation	Cryopreserved human liver microsomes	>100 μmol/l	Kopečná-Zapletalová et al. (2017)
3	3A4/3A2	Testosterone 6b-hydroxylation	Cryopreserved human liver microsomes	>100 μmol/l	Kopečná-Zapletalová et al. (2017)
		Midazolam 10-hydroxylation	Cryopreserved human liver microsomes	>100 μmol/l	Kopečná-Zapletalová et al. (2017)
		Nifedipine 4′-hydroxylation	Human liver microsomes	>50 μM	Arora et al. (2015)
			Rat liver microsomes	>50 μM	Arora et al. (2015)
4	2B6	7-ethoxy-4-(trifluoromethyl) coumarin 7-deethylation	Cryopreserved human liver microsomes	>100 μmol/l	Kopečná-Zapletalová et al. (2017)
5	2C8	Paclitaxel 6-hydroxylation	Cryopreserved human liver microsomes	>100 μmol/l	Kopečná-Zapletalová et al. (2017)
6	2C9/2C11	Diclofenac 40-hydroxylation	Cryopreserved human liver microsomes	>100 μmol/l	Kopečná-Zapletalová et al. (2017)
		Diclofenac 4-hydroxylation	Human liver microsomes	>50 μM	Arora et al. (2015)
			Rat liver microsomes	>50 μM	Arora et al. (2015)
7	2C19	S-mephenytoin 40-hydroxylation	Cryopreserved human liver microsomes	>100 μmol/l	Kopečná-Zapletalová et al. (2017)
8	2D6/2D4	Bufuralol 10-hydroxylation	Cryopreserved human liver microsomes	>100 μmol/l	Kopečná-Zapletalová et al. (2017)
		Dextromethorphan O-demethylation	Human liver microsomes	24.83 μM	Arora et al. (2015)
			Rat liver microsomes	>50 μM	Arora et al. (2015)

(continued)

Table 14.3 (continued)

S. No.	Enzyme subtype	Test compound	Model	IC50 value	References
9	2E1	Chlorzoxazone 6-hydroxylation	Cryopreserved human liver microsomes	>100 μmol/l, >50 μM	Arora et al. (2015), Kopečná-Zapletalová et al. (2017)
			Rat liver microsomes	>50 μM	Arora et al. (2015)

be an anti-fibrotic agent against carbon tetrachloride-induced hepatotoxicity in rats (Breikaa et al. 2013a). The BCA acts through multimechanistic pathway among which one of the targeted molecules is the CYP450 enzymes (CYP4502E1 and CYP4501A1) in conjugation with pro-inflammatory and pro-fibrotic mediators (Breikaa et al. 2013b). Moreover, after searching the database, there are very few findings in the last 10 years that could be found reporting the interaction of both FMN and BCA with the microsomal enzyme cytochromes (CYP450).

Furthermore, despite inhibition metabolic enzymes, few investigations also reported the induction of some cytochrome enzymes by FMN listed in Table 14.4. This contradictory research needs more data to confirm the exact role of bioflavonoids upon CYP enzymes. Hence, still the studies are required to explore the further action of FMN and BCA upon various microsomal enzymes.

14.5 Interaction with ABC Transporters

The most widely distributed and largest integral protein family known is the ABC (ATP-binding cassette) transporter present in the body. Presence of two nucleotide-binding domains and two transmembrane domains is the characteristic feature of ABC transporter. Binding of any exogenous and endogenous ligand to the transporter leads to the conformational changes in the nuclear-binding domain that further alters the transmembrane domains of the receptor. These rearrangements of the domains outturn in to the modulation of internal cytosolic and nuclear molecular messengers for initiation of signaling pathways. Further, an untoward activation or inhibition in this feature of ABC transport could lead to various types of side effects or adverse events and drug toxicity. Moreover, the potential involvement of ABC transporters against the drug-drug, herb-drug, and herb-herb interaction had been already established by many researchers.

Furthermore, FMN and BCA are two major isoflavonoids found in red clover extract. They are also highly recommended for their anti-osteoporotic activity. Hence, it could be possible that they are being administered with other co-prescribed allopathic medicines that are ABC transporter activators or inhibitors or the substrates. Therefore, it is important to know the effect of FMN and BCA towards ABC

Table 14.4 Modulation of various transporters due to FMN and BCA

S. No.	Drug	Isoflavonoid	Targeted transporter	In vitro/in vivo model	Consequences	References
1.	Raloxifene	BCA	UGT substrates	Gunn rats deficient in UDP-glucuronosyltransferase (UGT) 1A, Eisai hyperbilirubinemic rats (EHBRs), which hereditarily lack multidrug resistance-associated protein (MRP) 2 and wild-type rats	Raloxifene concentration was absorbed twice than the wild type rats	Kosaka et al. (2011)
2.	–	FMN	P-gp and BCRP	HepG2 and wild-type C57BL/6 mice and nuclear factor E2-related factor-2 knockout (Nrf2−/−) C57BL/6 mice	Upregulated the P-gp and BCRP	Lou et al. (2019)
3	–	FMN, BCA	OATP	MDCKII cells	Aglycones inhibited the transporter with Ki value from 1 to 20 μM	Navrátilová et al. (2018)
4	–	FMN	Pgp	Molecular docking (computational model)	High binding affinity towards transporter and its inhibition	Wongrattanakamon et al. (2017)
5	–	FMN	CYP3A4, CYP2B6, CYP2E1, UGT1A, UGT1A6, SULT1A1, and SULT1A3 P-gp, MRP2, BCRP, MRP1, and MRP3	HepG2 cell line	FMN-induced CYP3A4, CYP2B6, UGT1A, P-gp, MRP1, MRP2, and MRP3 protein expression but inhibited the expression of CYP2E1, SULT1A1, and SULT1A3	Zhang et al. (2016)
6	–	BCA	OATP1B1	HEK293 cell line	The transporter expression was inhibited thus inhibiting bosentan-induced liver injury	Fan et al. (2020)

(continued)

Table 14.4 (continued)

S. No.	Drug	Isoflavonoid	Targeted transporter	In vitro/in vivo model	Consequences	References
7	Doxorubicin, temozolomide, and mitoxantrone	BCA	BCRP	BCRP-MDCKII cell	Inhibition of BCRP-mediated efflux increased the doxorubicin and temozolomide concentration while increased the concentration of mitoxantrone	Fan et al. (2019)
8	Doxorubicin	BCA	Pgp	Doxorubicin-resistant K562 cells	Inhibited the transporter	Dash and Konkimalla (2017)

transporters. There are literatures published regarding the modulation of ABC transporter by the administration of isoflavonoids (Wongrattanakamon et al. 2017) in which the authors have performed the molecular docking, pharmacophore modeling, and molecular dynamic simulation studies for an ABC transporter, P-gp (P-glycoprotein), of mouse origin with some of the mostly utilized bioflavonoids. They have discerned the certainty of plausible interaction of these isoflavonoids with other co-prescribed drugs as they have the potential to inhibit the P-gp efflux mechanism. Therefore, it is a major concern when the P-gp substrate drug is co-administered with these isoflavonoids which might lead to the intracellular increase in concentration of the substrate drug, thereby altering its therapeutic index and safety profile. In a research, FMN has been investigated as a nephroprotective agent against cisplatin-induced renal toxicity by altering the expression of organic cation transporter 2 (OCT2) and multidrug resistance-associated proteins (MRPs). FMN is reported to enhance the expression of MRP gene whilst alleviating the OCT2 expression, hence decreasing the intratubular accumulation of cisplatin in kidney resulting in to the reduced nephrotoxicity of the drug (Huang et al. 2017). In a recent investigation, a group of researchers evaluated the inhibitory effect of 99 major flavonoids upon BCRP (breast cancer resistance protein) under both in vitro and in vivo experimental conditions using BCRP-associated MDCK II cells and rat as an animal model (Fan et al. 2019). Their findings linked to molecular docking analysis along with structural activity relationship could further assist in predicting the potential risk in interaction between the isoflavonoids and other co-administered drugs.

Further, it has also been observed that BCA along with ciprofloxacin could also inhibit the efflux pump of methicillin-resistant *Staphylococcus aureus* in a synergistic manner (Zou et al. 2014). The concentration of ciprofloxacin was found to be significantly increased by 83% at 15 min after combining with BCA which was similar to the effect of positive control drug, reserpine. BCA was also examined as a P-gp inhibitor after being formulated into a solid dispersion. When investigated under in vitro experimental conditions, it remarkably augmented the cellular uptake of a P-gp substrate, rhodamine123 in NCI/ADR-RES cells by 2-3 folds. They have also examined the BCA for its inhibitory efficacy after oral and intravenous administration of BCA with diltiazem (a P-gp substrate) and its metabolite desacetyldiltiazem to rats. Upon pharmacokinetic analysis, the AUC (area under the curve) of desacetyldiltiazem was found to be threefold without affecting the concentration of diltiazem. Therefore, when BCA is developed into a new formulation as solid dispersion, its inhibitory potency is enhanced. Moreover, in a study, Singh and his colleagues have utilized BCA as a P-gp and CYP inhibitor to investigate its effect on bioavailability of an anticancer P-gp substrate tamoxifen and its metabolite. The concentration of tamoxifen and its metabolite was found to be decreased suggesting the low bioavailability of tamoxifen owing to its characteristic of being the P-gp substrate (Singh et al. 2012).

Furthermore, after searching the database, there are only few publications regarding the pharmacokinetic and pharmacodynamic interactions of both the isoflavonoids with either CYP450 microsomal enzymes or ABC transporters that have been summarized in the aforementioned paragraphs and their respective tables.

14.6 Conclusion

A large number of literatures have been published regarding the pharmacological importance of both the isoflavones. Newer drugs and conventional therapies involving the use of plants and/their constituents have been continuously scrutinized against many disorders. Most of the plant-derived compounds constituting flavonoids are polyphenolic in nature. A large number of papers have been published confirming the health-related benefits of dietary flavonoids. Large-scale clinical trials are required to be conducted in order to establish the potential usefulness of flavonoids in the treatment of various disease conditions. This requires the development of rapid and validated assays for the characterization and quantification of the phytol-constituents and their metabolites in biological matrices. Plant extracts though contain a mixture of chemical constituents which complicates the process of bioanalysis required in the drug development process. The present chapter highlights the pharmacokinetic interaction of FMN and BCA involving the microsomal CYP enzymes, multi-mechanistic membrane ABC transporters resulting in to the altered bioavailability of other co-administered drugs. They are increasingly being examined for their beneficial effect against many diseases. Apart from their therapeutic importance, large scale of research is required to investigate their interaction and binding capacity towards metabolic enzymes and ABC transporters at preclinical and clinical level. This necessitates to study the pharmacokinetic effect of these phytoestrogenic compounds in order to overcome the adverse events and synergizing the therapeutic potency of isoflavonoids.

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Conflict of Interest The authors declare that there are no conflicts of interest.

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