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A GLIMPSE OF PHYTOPHARMACEUTIC RESEARCH AT THE ASTHI CENTRE

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Contents

Designed by- Mr. Kazim Raza

Introduction

Phytopharmaceutical assessment of Indian medicinal plants to prevent and treat metabolic bone diseases and fracture healing is a major area of research under the ASTHI program. We begin our screening for the osteogenic effect by making either ethanolic or ethyl acetate extract of specific part of a medicinal plant. The part of the plant is decided from its ethno-traditional use. Parameters of bone regeneration at the femur osteotomy site of adult rats are standard screening protocol that is completed in 2 weeks. We typically test doses between 250mg/kg to 1g/kg given by oral gavage daily. If a promising bone regeneration effect is observed, we next perform activity-guided fractionation to localize the most active fraction (MAF) with aim of reducing the dose by a minimum of 3X from the dose of the first extract. From MAF, we then isolate abundant compounds sufficient to test their effects in bone cells; osteoblasts, osteoclasts and MSCs. We also use these culture systems to understand the mechanisms of action the compounds using the state-of-the-art OMICs, bioinformatics-based pathway analysis and conventional studies related to cell signaling. The bioactive compounds (at least four) are used to standardize the extract/fraction through the principal component analysis (PCA).

Subsequently, we test the efficacy of the standardized extract/fraction in a variety of preclinical animal models of bone loss including bilateral ovariectomy (OVX), 5/6 nephrectomy (a model of secondary osteoporosis), glucocorticoid-induced osteoporosis and high fat diet-induced osteopenia. We also assess the most active compound/s obtained by in vitro screening in the above models. We also determine the preclinical PK of these compounds. After acquiring GLP accreditation, we have begun IND-enabling toxicity studies of extracts/fractions. Besides, we carry out IND-enabling pharmacokinetics studies. Taken together, we have all the expertise and facilities for phytopharmacutic development of a standardized plant extract/fraction from the discovery of its therapeutic effect in relevant disease model.

In this document, we included two categories of studies; a) discovery and products development and b) discovery and pipeline building. In the former category, we have four plants including *Dalbergia sissoo*, *Cassia occidentalis*, *Ulmus wallichiana* and *Butea monosperma*. In the latter category, we have nine plants including, *Cupressus sempervirens, Pterospermum acerifolium, Ginkgo biloba, Coelogyne cristata, Passiflora foetida, Pholidota articulate, Allophylus serratus*, *Cissus quadrangularis* and *Vitex negundo*. We will subsequently describe plants investigated in t hese two broad categories.

A. Discovery and Products Development

Chapter 1: *Dalbergia sissoo*

I. Introduction

Dalbergia sissoo Roxb. belongs to the legume family (Fabaceae), is a large deciduous perennial tree, growing widely in lowland region throughout India, Pakistan, Bangladesh, Afghanistan and Nepal. *D. sissoo*, popularly known as 'Indian rose wood' and 'Shesham' has been used as traditional medicine for a number of ailments. Sixteen isoflavones and flavonols with their glucosides, a lignan glucoside and an itaconic derivative were isolated from isolated from the ethanolic extract of *D. sissoo* leaves. These compounds include genstein, biochanin A, pratensein, biochanin 7-*O*-glucoside, biochenin A 7-*O*-[β-D-apiofuranosyl-(1→6)-β-D-glucopyranoside], biochenin A 7-*O* β-D-apiofuranosyl-(1→5)-β-D-apiofuranosyl-(1→6)-β-D-glucopyranoside], genistein 8-*C*-β-D-glucopyranoside, caviunin, caviunin 7-*O*-β-D-glucopyranoside, caviunin 7-*O*-[β-D-apiofuranosyl-(1→6)-β-D-glucopyranoside] (CAFG), kaempferol 3-*O*-β-D-glucopyranoside, kampferol-3-*O*-rutinoside, quercetin 3-*O*-β-D-glucopyranoside, quercetin 3-*O*-rutinoside, syringaresinol-4'-*O*-β-D-monoglucoside, dalsissooside and (E)-4-methoxy-2-(3,4-dihydroxybenzylidene)-4-oxobutanoic acid. Of these compounds, dalsissooside, (E)-4-methoxy-2-(3,4-dihydroxybenzylidene)-4-oxobutanoic acid and CAFG are novel compounds. All compounds were screened using in vitro osteogenic assay system which showed that genstein, biochanin A, pratensein, biochanin 7-*O*-glucoside and CAFG have osteogenic effect (1).

An ethanolic extract of *D. sissoo* leaves (250mg/kg p.o.) accelerates the process of regeneration of the woven bone tissue after drill-hole injury in femur to mimic fracture condition in rats (2). An extract made from the leaves and pods of *D. sissoo* (butanol-soluble standardized fraction [BSSF]) when tested in OVX rats, a model for postmenopausal osteopenia showed improved trabecular microarchitecture of the long bones, increase biomechanical strength parameters of the vertebra and femur, decreased bone turnover markers (osteocalcin and type I collagen) and decreased expression of skeletal osteoclastogenic genes, and increased new bone formation and expression of osteogenic genes in the femur compared with OVX rats received vehicle. There were four marker compounds present in BSSF including biochanin (0.7%), pratensein (0.4%), genistein (0.07%) and

CAFG (3.7%) (3).

noids with a 4-arylchroman-type structure that distinguishes them from flavonoids with 2-phenylchromen-4-one backbone. Neoflavonoids isolated from HEE include dalsissooal (a new compound), cearoin, dalbergin, 4-methoxy dalbergion, dalbergiphenol, dalbergichromene, methyl dalbergin and latinone. Of these compounds, dalsissooal, latinone, dalbergiphenol and dalbergin are abundant in HEE. In a rat femur osteotomy model, HEE at as low as 250mg/kg oral dose stimulated bone regeneration at the callus and accelerated healing which suggested its osteogenic role. Daily oral dosing of OVX osteopenic rats with HEE at 500mg/kg maximally recovered all trabecular parameters of femur and L5 over the OVX rats treated with vehicle. The underlying mechanism was a dual effect of stimulation of bone formation and suppression of bone resorption. HEE also recovered OVX-induced loss of femur bending strength. Finally, HEE's uterine and hepatic safety has been established (5).

Protection against osteoarthritis (OA) has been reported for an ethanolic extract of *D. sissoo* leaves (DSE) containing 0.02% biochanin A, 0.05% pratensin, 2.11% genistein, 2.48% biochanin A 7-*O*-b-D-glucosides and 2.5% CAFG. Monosodium iodoacetate (MIA)-induced OA in rats is significantly mitigated by DSE (250- and 500mg/kg p.o.) evident from maintenance of articular cartilage structure, morphology and architecture of the affected joint, and integrity of subchondral bone. Serum biomarkers of OA including CTX-II, IL-1β and MMPs were significantly reduced by DSE. At the cellular level, DSE supported chondrocyte differentiation and protected against IL-1β-induced apoptosis (4).

In Indian medicinal practice, the heartwood extract of *D. sissoo* also has medicinal property. Ethanolic extract of heartwood (HEE) contains neoflavonoids with a 4-arylchroman-type structure that distinguishes them from flavonoids with 2-phenylchromen-4-one backbone. Neoflavonoids isolated from HEE include dalsissooal (a new compound), cearoin, dalbergin, 4-methoxy dalbergion, dalbergiphenol, dalbergichromene, methyl dalbergin and latinone. Of these compounds, dalsissooal, latinone, dalbergiphenol and dalbergin are abundant in HEE. In a rat femur osteotomy model, HEE at as low as 250mg/kg oral dose stimulated bone regeneration at the callus and accelerated healing which suggested its osteogenic role. Daily oral dosing of OVX osteopenic rats with HEE at 500mg/kg maximally recovered all trabecular parameters of femur and L5 over the OVX rats treated with vehicle. The underlying mechanism was a dual effect of stimulation of bone formation and suppression of bone resorption. HEE also recovered OVX-induced loss of femur bending strength. Finally, HEE's uterine and hepatic safety has been established (5).

II. CAFG

CAFG is the most abundant compound present in BSSF and has in vitro osteogenic effect. In bone marrow stromal cells, CAFG stimulates osteogenic and chondrogenic differentiation that likely contributed to its ability to promote fracture repair in a rat femur osteotomy model. In osteoblasts,

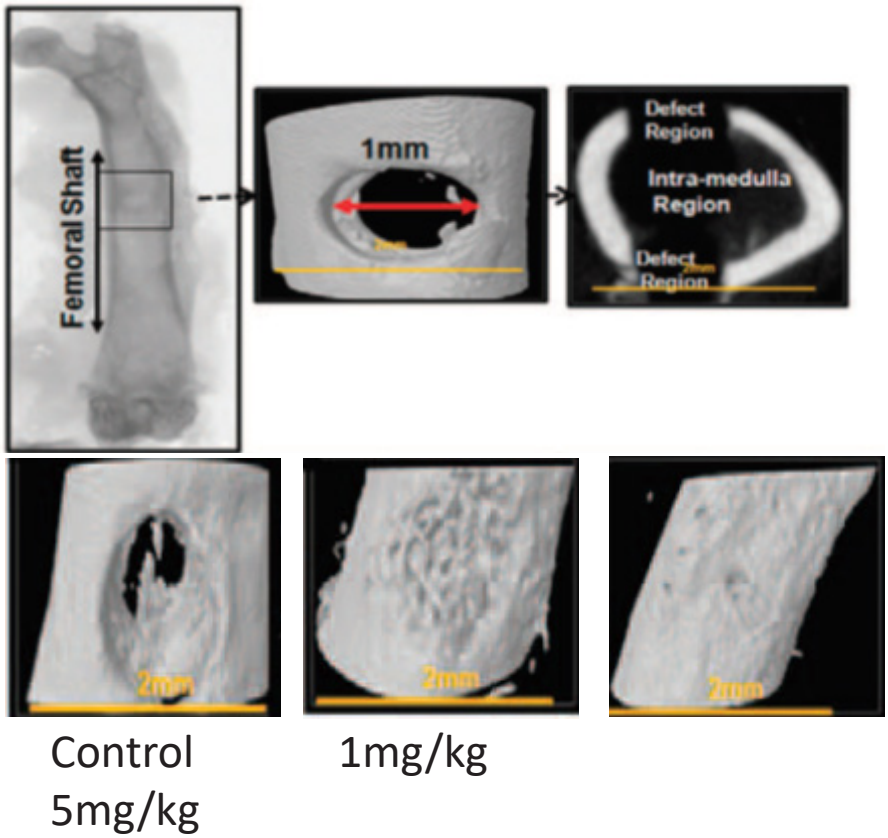

Fig 1. CAFG promotes bone regeneration in the drill-hole site in mice. Upper panel - 1 mm hole by drilling was generated at femur mid-diaphysis. Defect and intra-medullary regions are visible by μCT. Lower panel – healing of the defect after 21 days of CAFG treatment.

CAFG activates BMP receptor and initiates Smad-dependent signalling. After phosphorylation of Smad1, Smad4 binds to phospho-Smad1 and translocates to the nucleus which in turn transactivates TCF/LEF complex to promote transcription of osteogenic genes. Furthermore, CAFG increases OPG production from osteoblasts to inhibit osteoclastogenesis (6).

III. Dalbergiphenol

Daily oral administration of dalbergiphenol (1- and 5 mg/kg) mitigates OVX-induced deterioration of bone strength and maintains trabecular microarchitecture by stimulating osteoblast function without inducing uterine hyperplastic effect in mice (7).

IV. Dalsissooal

Daily oral administration of dalsissooal (1- and 5 mg/kg) mitigates OVX-induced deterioration of bone strength and maintains trabecular microarchitecture by stimulating osteoblast function without inducing uterine hyperplastic effect in mice (8).

V. Dalbergin

Dalbergin is osteoprotective in the therapeutic mode as it ameliorated the cancellous bone mass and quality and inhibited osteoclast differentiation without any harmful effect on liver and

uterine tissue in rodents (9).

VI. Clinical trials of *D. sissoo* extract

In a single arm, pilot clinical study, *D. sissoo* leaves extract (CAFG, 0.67%; biochanin 7-*O*-glucoside,1.5%; genistein, 0.75%; pratensein, 0.2%, and biochanin-A, 1%) was given to adults (18-60 years) of both sexes who suffered fracture. Fracture sites were humerus, ulna, fibula, radius and tibia. *D. sissoo* leaves extract was gives 300mg b.d. for 8 weeks. At the end of 8 weeks all fractures were healed and restored functional mobility. The extract was well-tolerated with no safety concern (10).

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XII. IPR

Maurya Rakesh, Preety Dixit, Ritu Trivedi, Vikram Khedgikar, Jyoti Gautam, Avinash Kumar, Divya Singh, Sheelendra Pratap Singh, Wahajuddin, Girish Kumar Jain, Naibedya Chattopadhyay. *Dalbergia sissoo* derived extract and compounds for the prevention of osteo-health related disorders designated as osteoNATURALcare. **European Patent (EP2705047 B1)**.

XIII. Commercialization

Marketed as Reunion® by Eris Lifesciences, Ahmedabad, Gujarat.

Chapter 2: *Cassia occidentalis*

"It is about a fourth-generation family practice from Puttur, a town in the southern state of Andhra Pradesh. In 1881, the first of their bone setters discovered a bone-healing herb by serendipity of sorts. While out hunting, Gopal Raju had caught a rabbit, breaking some of its bones in the process, and had wrapped the injured animal in leaves to take it home for the pot. But by the time he got there, the rabbit was able to walk, albeit with a severe limp. Suspecting the plant he had used was medicinal, Raju predesignated the rabbit from supper to his greatest experiment – he made a paste out of the leaves, applied it to the animal and reportedly watched it heal completely in a matter of days. Over subsequent years Raju experimented with chicken, calves and sheep, retrospectively incorporating ideas from Ayurveda's key text.

The herb is kept secret to this day by practitioners but has since been identified in a study by a taxonomist as Kasamadra, or Cassia occidentalis.

The story goes that he then became part of the First World War effort in India, when his services were employed by the British government for treating wounded soldiers and civilians. His brother's grandson, who had trained in general medicine, took up his great uncle's folk practice, opening a hospital that continues to be family run and now is one of two in the area, in addition to several smaller village clinics. Though the hospitals see more than 300 patients a day, the Kasamadra is still only gathered from the places in which it grows wild."

Bone-setter's Waiting Room: Travels Through Indian Medicine by Aarathi Prasad published in 2016 by Profile Books Ltd., London, U.K.

I. Introduction

C. occidentalis belongs to family Ceasalpiniaceae is a common weed distributed from foot hills of Himalaya to West Bengal, South India, Burma & Sri Lanka. It is an annual, erect undershrub. Ethanolic extracts from leaf and stem of *C. occidentalis* were prepared and their efficacy tested in rat femur osteotomy (fracture healing) model. Subsequently, a butanolic fraction was prepared and osteogenic efficacy compared with the ethanolic extract, and upon finding the former to be more potent, its osteogenic effect was studied in details in glucocorticoid-induced osteoporosis (GIO model). After chemical finger-printing, 10 compounds were isolated (**Table 1**) and their osteogenic effect was assessed on rat primary osteoblast culture.

Table 1: Osteogenic activity of isolated compounds based on ALP assay in RCO

Name of the compound	Activity (EC_{50})
Apigenin (1)	768.05±54.45 nM
4-Methoxy-2´,4´-dihydroxy chalcone (2)	-
4´,7-Dihydroxyflavone (3)	26.32±16.97 nM
Luteolin (4)	16.32±11.97 nM
3´,4´,7-Trihydroxyflavone (5)	14.8±9.19 nM
Emodin (6)	33.27±11.64 nM
Nicotinic acid (7)	-
Chrysophanol-1-O-β-Gentiobioside (8)	-
Rhamnocathartin (9)	-
Apigenin-6C-glycoside (10)	620.93±105.6 nM

"-": no activity

Our study found that a standardized extract of an ethanolic extract and its butanolic fraction from the stem of *C. occidentalis* has bone anabolic as well as anti-catabolic effects on skeleton, thereby affording protection to GIO (1).

To assess whether CSE and CSE-Bu had bone anabolic effect, surface referent bone formation was determined by calcein double labelling study in femur diaphysis. MP treatment significantly reduced periosteal (p)-mineralizing surface per bone surface (pMS/BS, an index of osteoblast activity), periosteal (p)-mineral apposition rate per bone surface (pMAR, average rate of osteoblast activity at each basic multicellular unit of remodeling site) and periosteal (p)-bone formation rate per bone surface (pBFR/BS, represents cumulative bone formation). Both CSE and CSE-Bu increased these three parameters over the MP group but were less than the control (Fig 1).

II. An oral formulation to enhance the efficacy of CSE-Bu

Although CSE-Bu provided significant protection against the methylprednisolone (MP)-induced bone loss (GIO), however, the protection was not complete, which suggested scope for improving its efficacy. Self-nano emulsifying drug delivery system (SEEDS) is an efficient approach for enhancing intestinal absorption of hydrophobic compounds that are present in CSE-Bu, leading to their improved bioavailability and more consistent temporal profile of their absorption. CSE-Bu contains six osteogenic compounds out of which isovitexin had the best osteogenic effect in vitro. Here, we developed a lipid-based SEDDS of CSE-Bu (CSE-Bu formulation denoted henceforth as CSE-BuF) to enhance absorption and consequently bioavailability of the osteogenic compounds present in CSE-Bu (2).

The globule size of CSE-BuF was in the range of 100–180nm of diluted emulsion and the zeta potential was -28 mV. CSE-BuF enhanced the circulating levels of five osteogenic compounds compared to CSE-Bu. CSE-BuF (50mg/kg) promoted bone regeneration at the osteotomy site and

completely prevented MP-induced loss of bone mass, deterioration of microarchitecture and strength by concomitant osteogenic and anti-resorptive mechanisms. The MP-induced downregulation of miR29a (the positive regulator of the osteoblast transcription factor Runx2) and miR17 and miR20a (the negative regulators of the osteoclastogenic cytokine RANKL) in bone was prevented by CSE-BuF. In addition, CSE-BuF protected rats from the MP-induced sarcopenia by downregulating the skeletal muscle atrogenes (Fig 2), adverse changes in body weight and composition. These effects of CSE-BuF were achieved without affecting the anti-inflammatory effect of MP. We conclude that CSE-BuF is an effective pharmacotherapy for mitigating the osteo-sarcopenic impact of chronic GC treatment without hindering the anti-inflammatory action of GC, and thus should be further developed for clinical translation (2).

III. Simultaneous determination of osteogenic compounds in serum

A sensitive and short LC-ESI-MS/MS method was developed and validated for the simultaneous determination of the concentrations of apigenin, apigenin-6*C*-glycoside, luteolin, trihydroxy flavone and emodin in rat plasma with shorter run time. The novel method was utilised for the evaluation of the oral pharmacokinetics after the administration of ethanolic extract of *C. occidentalis* leaves to rats (3).

After the oral administration of the extract, apigenin, luteolin, trihydroxy flavone and emodin appeared to be absorbed rapidly compared to apigenin-6*C*-glycoside. A second peak was observed in

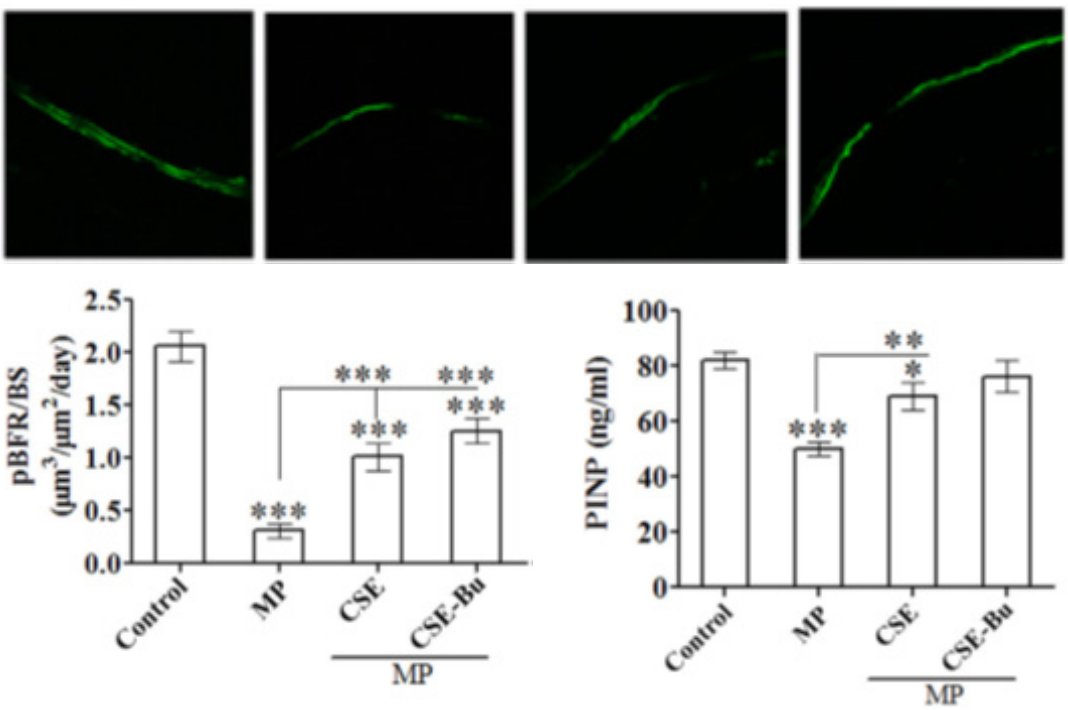

Fig 1. CSE and CSE-Bu stimulate bone formation and CSE-Bu inhibits bone resorption. Surface referent bone formation parameters were determined at femur diaphysis in response to indicated treatments. Upper panel is showing representative photomicrographs of calcein labeling and lower panel showing quantification of various bone formation parameters. Data are expressed as mean ± SEM (n = 10/group) *P < 0.05, **P < 0.01 and ***P < 0.001 compared to control or as indicated.

the plasma pharmacokinetic profiles of flavones which might be due to entero-hepatic recirculation as reported earlier in case of flavanoids. Apigenin-6*C*-glycoside and trihydroxy flavone were eliminated within 12 h and luteolin and emodin were eliminated within 24 h. Apigenin could be detected in plasma until 8 h. The pharmacokinetic profiles of apigenin-6*C*-glycoside is markedly different from other analytes, possibly owing to the presence of a sugar moiety in this molecule.

In rat liver microsome, >90% of apigenin and emodin underwent phase I metabolism within 60 min whereas 60% and 30% in the cases of apigenin-6*C*-glycoside and trihydroxy flavone, respectively. Luteolin did not undergo phase I metabolism in rat liver microsome. The in vitro stability studies showed that luteolin was relatively stable in different conditions and this might be the reason for its abundant plasma levels (high C_{max} and AUC values) in the pharmacokinetic study. The results are useful for further pharmacodynamics investigation of the ethanolic extract of *C. occidentalis* leaves and the bioanalytical method may be applicable for phase 1 clinical trial according to phyto-pharmaceutical guideline of DCG(I) (3).

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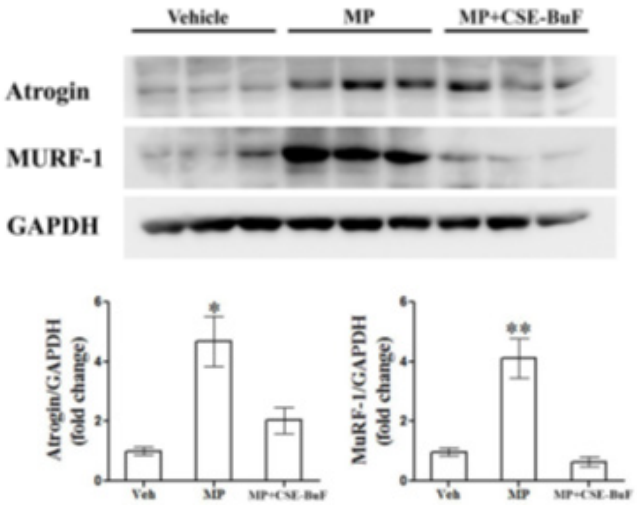

Fig 2. CSE-BuF prevented MP-induced muscle atrophy. Western blot images and densitometric quantification of bands of atrogin-1 and MuRF-1 expression in gastrocnemius muscle from rats of the indicated groups are shown. Data are expressed as mean ± SEM (n = 6/group) *P < 0.05, **P < 0.01 and ***P < 0.001 compared to vehicle (veh).

be detected in plasma until 8 h. The pharmacokinetic profiles of apigenin-6*C*-glycoside is markedly different from other analytes, possibly owing to the presence of a sugar moiety in this molecule.

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V. Herb-drug interaction studies

We investigated the effect of ethanolic extract of *C. occidentalis* leaves on the pharmacokinetics of the drugs that are prescribed to patients suffering from autoimmune diseases with high inflammatory burden including acetaminophen (ACET), theophylline (THEO), omeprazole (OMEP), methotrexate (MTX), and methylprednisolone (MP) in SD rats. The extract showed pharmacokinetic interaction with ACET, MTX and MP. No significant interaction of EECO was observed when co-administered with THEO and OMEP. The in vitro study suggested that EECO inhibited the CYP1A2, CYP2C9 and CYP3A4. Therefore, drugs which are metabolized by these enzymes when co-administered with the ethanolic extract of *C. occidentalis* leaves could be prone to herb–drug interaction and dose adjustment may be required. These results will be helpful for conducting the phase I clinical trials according to the guideline of DCG(I) for the phytopharmaceutical development (4).

VI. Preclinical safety pharmacology and toxicity assessments in rodents

We showed that the ethanolic extract of the leaf and stem of *C. occidentalis* has osteogenic effect and mitigates GIO at 250mg/kg dose. We next studied the preclinical safety and toxicity of this extract. The extract was prepared as per regulations of Current Good Manufacturing Practice for human pharmaceuticals/phytopharmaceuticals and all studies were performed in rodents in a GLP-accredited facility. In acute dose toxicity as per New Drug and Clinical Trial Rules, 2019 (prior name schedule Y), in rats and mice and ten-day dose range-finding study in rats, the extract showed no mortality and no gross abnormality at 2500 mg/kg dose. Safety Pharmacology showed no adverse effect on central nervous system, cardiovascular system, and respiratory system at 2500 mg/kg dose. The extract was not mutagenic in the Ames test and did not cause clastogenicity assessed by in *vivo* bone marrow genotoxicity assay. By a subchronic (90 days) repeated dose (as per OECD, 408 guideline) study in rats, the no-observed-adverse-effect-level was found to be 2500 mg/kg assessed by clinico-biochemistry and all organs histopathology. We conclude that the extract is safe up to 10X the dose required for its osteogenic effect (5).

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Pal S and Chattopadhyay N et al. Bioactive extract, fraction of *Cassia occidentalis* and formulation thereof for bone regeneration and treatment of glucocorticoid-induced musculoskeletal disease. Filed on 08.06.2018, **application number 201811021504**.

VII. Licensing status

Licensed to Pharmanza Herbals Pvt. Ltd, Gaujarat.

Chapter 3: *Ulmus wallichiana*

I. Introduction

Ulmus wallichiana Planchon, belongs to the family Ulmaceae, distributed through Himalayas from Afghanistan to W. Nepal. In India this plant is found in Kumaon and Garhwal Himalaya, locally calledβ as Chamarmou, In and around Kumaon traditional healers use this plant for promoting fracture healing but the effects on osteoporosis and total osteo-health and related disorders have not been scientifically explored. Fractionation of ethanolic extract of stem bark of *U. wallichiana* led to the isolation of two new flavonoid *C*-glucosides, five known flavonoid *C*-glucosides and one new phenolic-*C*-glucoside. Four marker compounds of the extract include quercetin-6-*C*-β-D-glucopyranoside (***1***), (2S,3S)-(+)-3',4',5,7-tetrahydroxydihydroflavonol-6-*C*-β-D-glucopyranoside (***2***), naringenin-6-*C*-β-D-glucopyranoside (***3***) and (2S,3S)-(+)-4',5,7-trihydroxydihydroflavonol-6-*C*-β-D-glucopyranoside (***4***) (1). Total ethanolic extract (TEE) and a butanolic fraction (BF) were tested for their osteogenic effect in growing rats and in adult OVX rats. Both were found to be effective in stimulating bone regeneration although the latter was 15X more potent (750 mg/kg TEE vs 50 mg/kg BF). Significant enrichment of compounds 1, 3 and 4 in the BF appears to have robustly potentiated its effect over TEE. Neither TEE nor BF has uterine estrogenicity (2).

II. Quercetin-6-*C*-β-D-glucopyranoside (QCG)

QCG was in the order of 10^4-fold and 10^2-fold more potent than quercetin in respectively inhibiting osteoclast formation from bone marrow precursor cells and osteoblast differentiation from bone marrow stromal cells. In a preventive model of postmenopausal osteopenia in rats, QCG (5mg/kg) was more potent than quercetin (20 mg/kg) in conserving bone assessed by suppression of bone remodelling through bone turnover markers, BMD, μCT and bone strength. QCG also stimulated the parameters of peak bone mass in growing rats which indicated its positive effect on modelling-directed bone formation. Since modelling-directed bone formation could promote bone formation in osteoporotic condition, we studied the effect of QCG in osteopenic OVX rats. QCG treatment increased bone formation rate and improved trabecular microarchitecture in osteopenic rats. Comparison with

the sham group (ovary intact) revealed complete restoration of trabecular bone in osteopenic rats treated with QCG. QCG levels in the bone marrow were ~50% of that of the plasma levels (3).

III. Quercetin-6-C-β-D-glucopyranoside

We compared the in vitro osteoblast differentiation effect of NCG with four natural analogs including naringenin, isosakuranetin, poncirin and phloretin. Acute dosing of NCG to new born mice at as low as 1mg/kg increased ER and ER and its downstream target gene BMP-2 in preosteoblast rich calvarium which suggested its osteogenic action via the ERs. Indeed, the differentiation promoting effect of NCG in osteoblasts was abolished by an ER antagonist, ICI 182780. In a reporter-based assay, NCG transactivated ERβ but not ER. In osteopenic OVX mice, NCG at 5mg/kg restored trabecular bone on a par with PTH. More strikingly, the bone formation rate that was significantly impaired in the OVX mice was completely restored by NCG to the control (ovary intact) level. At 5mg/kg dose, naringenin failed to restore OVX-induced bone loss which suggested that NCG was more potent than naringenin. Additional notable differences include 1.5-fold greater bioavailability of NCG than naringenin and ER selective osteogenic action of NCG. Despite signaling by ER in osteoblasts, NCG has no uterine estrogenicity thus implying a SERM-'like' action (4).

IV. (2S,3S)-Aromadendrin-6-C-β-D-glucopyranoside (ACG)

ACG is a novel compound belonging to flavonol class. At nanomolar concentrations, ACG increased differentiation of preosteoblasts obtained from neonatal mouse calvaria. The gene expression of osteogenic markers, including Runx-2, BMP-2, type I collagen and osteocalcin were elevated in the preosteoblasts. The extracellular matrix mineralization was higher in preosteoblast and bone marrow cells when AG was present in the medium. Furthermore, ACG protected the differentiated osteoblasts from serum deprivation-induced apoptosis, and increased the expression of the anti-osteoclastogenic cytokine, OPG. It inhibited osteoclast differentiation of bone marrow precursor cells to osteoclasts in the presence of RANKL and monocyte/macrophage-colony stimulating factor (M-CSF). Additionally, in 3T3-L1 preadipocytes, ACG decreased the expression of genes involved in adipogenesis, including peroxisome proliferator-activated receptor gamma (PPARγ), sterol regulatory element binding protein (SREBP) and CCAAT/enhancer-binding protein alpha (CEBP/α), and induced apoptosis of differentiated adipocytes. Induction of adipogenic differentiation was also inhibited in the presence of AG. AG exhibited no estrogenic/antiestrogenic effect (5). Form these data, it is concluded that ACG has potent osteogenic, anti-osteoclastogenic and antiadipogenic effects, which may translate to a better skeletal outcome in postmenopausal osteoporosis.

V. 6-*C*-β-D-glucopyranosyl-(2S,3S)-(+)-3′,4′,5,7-tetrahydroxy-flavanone (GTDF)

GTDF is another novel compound isolated from the stem-bark of *U. wallichiana*. GTDF stimulated osteoblast proliferation, survival, and differentiation but had no effect on osteoclastic or adipocytic differentiation. Two mechanisms underlying the action of GTDF in osteoblasts. In cultured osteoblasts, GTDF transactivated the aryl hydrocarbon receptor (AhR). Activation of AhR mediated

the stimulatory effect of GTDF on osteoblast proliferation and differentiation (6). Furthermore, GTDF stimulated cAMP production, which mediated osteogenic gene expression. We next observed that GTDF binds to adiponectin receptors (AdipoRs) (7). In this regard, GTDF has 70-fold higher affinity to AdipoR1 than AdipoR2. Activation of AdipoRs is known to increase insulin sensitivity. Given such mechanisms of action, i.e. stimulation of various events of osteoblast lifespan and stimulation of AdipoRs led us to investigate the skeletal effects of GTDF in preclinical models that mimic postmenopausal osteoporosis and diabetes-induced bone loss (8).

In preventive mode, GTDF (5mg/kg) mitigated OVX-induced deterioration of bone strength and maintains trabecular microarchitecture without a uterine hyperplastic effect (9). In therapeutic mode, i.e. in OVX osteopenic rats, GTDF had osteoanabolic effect on a par with PTH (6). In osteopenic rats with femur osteotomy, GTDF showed accelerated healing response through new bone formation. The osteoanabolic effect of GTDF in osteopenic rats was translated to greater bone strength (6). In adult rats, GTDF however has a low bioavailability that was found to be 1.04% (9).

Moreover, since corticosteroids cause osteoblast apoptosis leading to osteoporosis and GTDF promotes osteoblast survival, we hypothesized that the compound could protect against corticosteroid-induced osteoporosis. We observed that (a) GTDF prevented development of osteopenia induced by dexamethasone and methylprednisolone wherein both trabecular and cortical bones were protected, (b) bone quality as assessed biomechanical strength was maintained by GTDF, (c) bone formation rate and marker (osteocalcin) were increased by GTDF over GC treatment, (d) GTDF protected GC-induced osteoblast apoptosis in vitro and in vivo, and (e) the mechanism involved inhibition of p53 and activation of AKT (10).

Consistent with its AdipoR agonistic property, GTDF induced adiponectin-associated signaling and enhanced glucose uptake and fatty acid oxidation in vitro, which were augmented or abolished by AdipoR1 overexpression or silencing, respectively. GTDF improved metabolic health, characterized by elevated glucose clearance, β-cell survival, reduced steatohepatitis, browning of white adipose tissue, and improved lipid profile in an AdipoR1-expressing but not an AdipoR1-depleted strain of diabetic mice (7). The discovery of GTDF as an adiponectin mimetic provides a promising therapeutic tool for the treatment of metabolic diseases associated with type 2 diabetes mellitus (T2D). T2D is associated with increased fracture risk and delayed facture healing; the underlying mechanism, however, remains poorly understood. We systematically investigated skeletal pathology in leptin receptor–deficient diabetic mice on a C57BLKS background (db). Compared with wild type (wt), db mice displayed reduced peak bone mass and age-related trabecular and cortical bone loss. Poor skeletal outcome in db mice contributed high-glucose–and nonesterified fatty acid–induced osteoblast apoptosis that was associated with peroxisome proliferator–activated receptor-α coactivator 1-α (PGC-1α) downregulation and upregulation of skeletal muscle atrogenes in osteoblasts. Osteoblast depletion of the atrogene muscle ring finger protein-1 (MuRF1) protected against gluco- and lipotoxicity-

induced apoptosis. Osteoblast specific PGC-1α upregulation by GTDF as well as metformin in db mice that lacked AdipoR1 expression in muscle but not bone restored osteopenia to wt levels without improving diabetes. Both GTDF and metformin protected against gluco- and lipotoxicity-induced osteoblast apoptosis, and depletion of PGC-1α abolished this protection. Although AdipoR1 but not AdipoR2 depletion abolished protection by GTDF, metformin action was not blocked by AdipoR depletion (8). We conclude that PGC-1α upregulation in osteoblasts by GTDF could reverse T2D–associated deterioration in skeletal health (Fig 3).

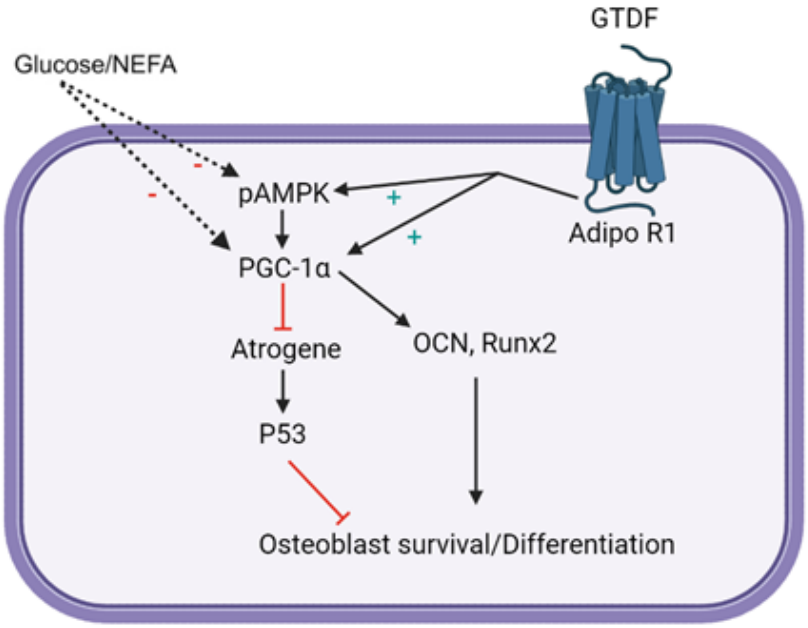
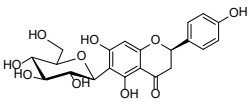
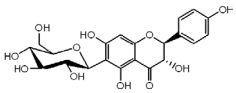
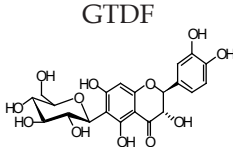
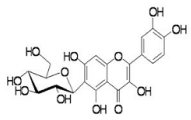


Fig 3. Schematic diagram summarizing the pathophysiological mechanisms of bone loss in db mice and a potential osteoanabolic role of AdipoR1. In osteoblasts, gluco- and lipotoxicity induce downregulation of pAMPK and PGC-1α (a key regulator of cellular energy metabolism whose expression and activity are modulated by AdipoR1) and upregulation (+) of several skeletal muscle atrogenes (atrophy-related genes involved in protein catabolism), resulting in impaired survival and differentiation of these cells. Osteopenia in db mice is associated with reduced expression of osteogenic genes likely due to decreased PGC-1α and increased atrogenes. Atrogenes could in turn activate p53, leading to inhibition of osteoblast survival and function. Osteopenia in db mice was reversed by GTDF (an AdipoR1 agonist) through PGC-1α to promote osteogenic genes and suppression of atrogenes that ultimately improved skeletal health by an osteoanabolic mechanism. Red line, inhibition.

Table 1. Salient features of bioactive compounds from *U. wallichiana*

Compounds	Osteogenic concentration	In vitro effects	Signaling pathway	In vivo effects	PK parameters	
					$AUC_{(0\text{-}24\ h)}$ ($ng.ml^{-1}.h^{-1}$)	Absolute bioavailability
NCG 	10^{-8}M	Differentiation & mineralization	Predominantly ERβ	Protects bone micro-architecture equivalent to E2	-	-
AG 	10^{-9}M	Differentiation & mineralization	-	-	-	-
GTDF 	10^{-8}M	Proliferation, differentiation & mineralization	AhR and AdipoRs	Improves bone micro-architecture, new bone formation and bone strength equivalent to PTH	132.46 ± 24.77	1.04%
QCG 	10^{-8}M	Differentiation & mineralization	AhR	Improves bone micro-architecture, new bone formation and bone strength equivalent to PTH	15,853 ± 103.83	27.7%

VI. Summary

Table 1 shows the salient features of the bioactive compounds in *U. wallichiana*'s stem-bark extract. From their in vitro effects, dependence on signaling pathways and in vivo effects, it appears that the extract through the synergistic and/or additive mode of action of these compounds has salutary effects in bone.

VII. References

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achievement and have non-estrogenic osteoprotective effect. ***Menopause*** 17:393-402, 2010.

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10. A novel flavonoid *C*-glucoside from *Ulmus wallichiana* preserves bone mineral density, microarchitecture and biomechanical properties in the presence of glucocorticoid by promoting osteoblast survival: a comparative study with human parathyroid hormone. ***Phytomedicine*** 20:1256-66, 2013.

XI. IPR

Rakesh Maurya, Preeti Rawat, Kunal Sharan, Jawed Akhtar Siddiqui, Gaurav Swarnkar, Geetanjali Mishra, Lakshmi Manickavasagam, Girish Kumar Jain, Kamal Ram Arya, Naibedya Chattopadhyay. *Ulmus wallichiana* Planchon derived extract and compound employed in prevention

or treatment of osteo-health related disorders. **United States Patent (US 8,669,232)**.

XII. Licensing

Kemextree, LLC, New Jersey, USA.

Chapter 4: *Butea monosperma*

I. Introduction

Butea monosperma (Lam.) Taub (Syn. *Butea frondosa*; Family Fabaceae) popularly known as Flame of the Forest, *Dhak*, *Palas* or 'Bastard teak' has proven to be a source of constitutive osteogenic agents belonging to isoflavonoid and pterocarpan groups. Ethanolic extracts of leaves, twigs, flowers, seeds and stem bark of *B. monosperma* were evaluated for in vitro osteogenic activity using neonatal rat calvaria derived primary osteoblast cultures. Of these, only the ethanolic extract of stem-bark showed significant osteogenic activity. Owing to such activity, an ethanolic extract made from the stem-bark of B. monosperma protected ovariectomized (OVX) rats from the loss of bone mass, deterioration of bone microarchitecture and bone strength (1).

Subsequently, a bioassay-guided fractionation of various solvents including butanolic, acetone and chloroform enabled isolation of ten isoflavones, four coumestans, two pterocarpans, one flavanonol, two triterpenes and three fatty esters (2). Of these compounds, the skeletal effect and action mechanisms of isoflavones including cajanin, cladrin, formononetin, isoformononetin and prunetin, and a pterocarpan, medicarpin have been extensively studied. Besides, oral bioavailability of several compounds have been determined in rodents.

II. Cajanin (2′-hydroxy-7-methoxy genistein)

Cajanin exerts mitogenic effect by increasing the number of osteoblasts at S- and G2/M phases of cell cycle. Cajanin also stimulates osteoblast differentiation evident from increased ALP mRNA levels and activity as well as formation of mineralized nodules from bone marrow stromal cells. These effects of cajanin are regulated by sequential activation of MEK-Erk and Akt pathways in osteoblasts. At 10mg/kg oral dose, cajanin enhanced bone mass, new bone formation and bone strength in growing rats. Cajanin has no estrogenic or anti-estrogenic effect in uterus and does not transactivate estrogen receptors in reporter-based assays (3).

III. Isoformononetin (7-methoxy daidzein)

Isoformononetin protects osteoblasts from serum deprivation-induced apoptosis by synergistic activation of MEK-Erk and Akt pathways. Isoformononetin also stimulates osteoblast differentiation by parallel activation of MEK-Erk and Akt pathways. At 10mg/kg oral dose, isoformonetin enhanced

bone mass, new bone formation and bone strength in growing rats (3). In a preclinical model of postmenopausal osteoporosis obtained by bilateral ovariectomy (OVX) of skeletally mature rats, isoformonetin dose-dependently (from 1- to 25mg/kg doses) restored trabecular microarchitecture, increased new bone formation, increased the serum osteogenic marker (procollagen N-terminal propeptide), decreased resorptive marker (urinary C-terminal teleopeptide of type I collagen) and diminished osteoblast apoptosis in bone (4). At the most effective osteogenic dose of isoformononetin (25mg/kg), plasma and bone marrow levels were comprised of ~90% isoformononetin and the rest, daidzein. Isoformononetin attained sufficient bone marrow levels to confer osteogenic effect in vivo (5).

Isoformononetin also reverses aberrant state of immunoactivation in estrogen deficient conditions characterized by increased production of IL-17-secreting Th17 cells and B-cell lymphopoesis, resulting in enhanced osteoclastogenesis and inhibition of osteoblastogenesis, ultimately leading to bone loss. Suppression of Th17 cells by isoformononetin is accompanied by increased production of regulatory T cells (Treg) (6).

Isoformononetin has no estrogenic or anti-estrogenic effect in uterus and does not transactivate estrogen receptors in reporter-based assays (4).

IV. Formononetin (4′-methoxy daidzein)

Fromononetin stimulates osteoblast differentiation via the p38 MAPK (7). In growing rats, formononetin at 10mg/kg oral dose increased bone mass at femur, tibia and lumbar vertebra (7). Formononetin reverses bone loss by increasing trabecular density in OVX rats with established osteopenia by stimulating osteoblast function in vivo (8). Fracture healing in osteoporotic individuals is compromised due to reduced osteoblast function. Hence, the efficacy of formononetin in healing osteotomy in OVX mice was assessed and the data showed that the compound markedly enhanced the healing by stimulating bone regeneration on a par with teriparatide, the osteoanabolic drug (9). Similar to isoformononetin, formononetin also reverses the aberrant immunoactivation in OVX mice and mitigates the resultant augmented systemic inflammation and in the process mitigates bone loss (6). In 3T3-L1 preadipocytes, formononetin activates the AMPK/β-catenin pathway and increases energy expenditure via up-regulation of uncoupling protein 1 (UCP1), and protects mice against obsesity-induced bone loss (10).

Parallel artificial membrane permeability assay (PAMPA) permeability of formononetin was found to be high. The oral pharmacokinetics of formononetin in rats indicated that it was rapidly absorbed into the systemic circulation. The rapid absorption of formononetin may be due to the high permeability and lipophilic nature. The oral bioavailability of unchanged/free formononetin is very low (~3%) and this may be due to extensive first-pass metabolism by phase I oxidative metabolism and phase II glucuronidation and/or sulfation in the intestine as well as in the liver (11). Formononetin has an absolute bioavailability of 4.29% in adult rats (7). Formononetin was extensively converted to its

metabolites daidzein and formononetin conjugates (glucuronides and/or sulfates) which appeared in the systemic circulation from the first sample point onwards. The RBCs uptake of formononetin was independent of the initial rat blood concentrations and time. The plasma protein binding of formononetin was found to be more than 93% (11).

CYP1A2 is much more susceptible to the inhibitory effects of formononetin compared to other CYPs. Formononetin also has moderate inhibitory effect on human CYP2D6. Thus, formononetin can be regarded as safe with minimum possibility of alteration in the pharmacokinetics of co-administered drugs, thus precluding the possibility of any adverse drug reactions due to enzyme inhibition at the hepatic level. However, the probability of pharmacokinetic interaction at the intestinal level must be carefully considered before co-administration of formononenetin with other drugs (12).

V. Cladrin (3′,4′-dimethoxy daidzein)

Cladrin stimulates osteoblast proliferation and differentiation by MEK-Erk pathway (7). At 10mg/kg p.o. dose treatment to growing rats, cladrin stimulates peak bone mass and bone formation over the control (7). In adult osteopenic OVX rats, cladrin at 10mg/kg dose had the following effects: (a) improved trabecular microarchitecture at the appendicular and axial sites that were mostly comparable with the sham group and was on a par with PTH, (b) increased mineral accrual and bone formation rates, cortical thickness, expression of the osteogenic genes in the long bones and serum PINP (osteogenic marker) levels to levels the sham group, (c) reduced OVX-induced urinary CTx (anti-resorptive marker) to the sham levels, and (d) maintained the positive skeletal effects after treatment discontinuation and in this regard, cladrin (10mg/kg) was equivalent to PTH whereas E2 effect waned. Despite having a phytoestrogen-“like” structure, cladrin had no estrogenic effect on the uterus (13).

The absolute bioavailability of cladrin in adult rats at 5, 10 and 20 mg/kg was found to be 16.58, 19.04 and 6.76%, respectively. It was also found to have high plasma protein binding and less or no affinity for blood corpuscles (14). Unlike formononetin, cladrin does not biotransform to daidzein or equol (7).

VI. Prunetin (4′,5-dihydroxy-7-methoxyisoflavone)

Prunetin stimulated proliferation and differentiation of osteoblasts by specifically activating a G-protein-coupled receptor, GPR30/GPER1. Rapid stimulation of GPR30 by prunetin results in the production of cAMP and activation of Erk/MAPK that subsequently led to upregulation of Runx2 followed by GPR30 proteins in osteoblasts. Stimulation of this osteogenic signaling resulted in enhanced bone regeneration at the fracture site by prunetin (15).

VII. Medicarpin (3-hydroxy-9-methoxypterocarpan)

Medicarpin is a pterocarpan-type phytoalexin which is also classified as methoxylated isoflavonoid. Medicarpin stimulated osteoblast differentiation and mineralization at as low as 100pM.

Studies with signal transduction inhibitors demonstrated involvement of a p38 mitogen activated protein kinase-estrogen receptor (ER)-bone morphogenic protein-2 pathway in mediating medicarpin action in osteoblasts. Co-activator interaction studies demonstrated that medicarpin acted as an ER agonist; however, in contrast to 17β-estradiol, medicarpin had no uterine estrogenicity and blocked proliferation of MCF-7 cells. Med increased protein levels of ERβ in osteoblasts. Selective knockdown of ERα and ERβ in osteoblasts established that medicarpin promotesteoblast differentiation via ERβ. Although, signaled through ERs in osteoblasts, medicarpin had no estrogenic effect in uterus (16).

Medicarpin also promotes osteoblast survival by inhibiting endoplasmic reticulum stress by targeting GRP78 which is an endoplasmic reticulum chaperone with anti-apoptotic property (17). Besides, medicarpin via ERs inhibits the production of TNF and the production of its downstream inflammatory cytokines, which appear to inhibit receptor activator of nuclear factor kB (RANKL): osteoprotegerin (OPG) ratio. RANKL: OPG ratio is the strongest regulator of osteoclastogenesis and medicarpin suppresses osteoclastogenesis by inhibiting this ratio beside exerting a direct inhibitory effect on osteoclastogenesis (18).

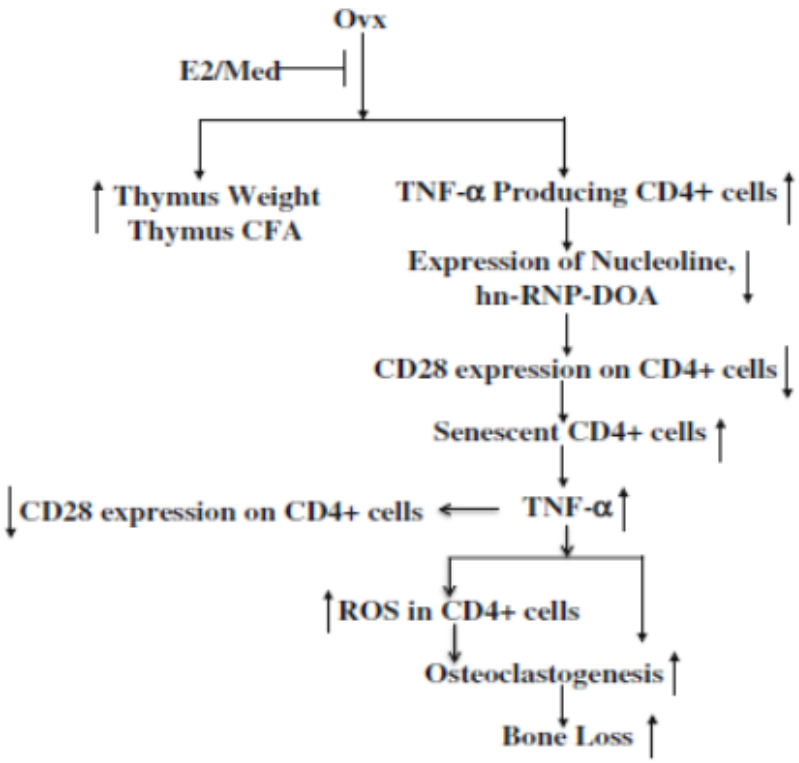


Fig 1. Schematic diagram showing various OVX-activated immunological alterations contributing to bone loss that are reversed by medicarpin. (1) OVX (E2 deficiency) increased thymus weight and cortical fraction area, (2) OVX increased TNF-α-producing T cells in the BM, (3) OVX reduced expression of nucleoline and hnRNP-D0A in the BM T cells, (4) OVX downregulates the expression of CD28 on T cells, and (5) OVX results in the production of more senescent T cell population that produce more TNF-α thus leading to decreased CD28 expression on $CD4^+$ T cells and enhanced osteoclastogenesis. Medicarpin reverses events 1-5.

In growing rats, medicarpin at 1- and 10mg/kg doses increased formation of osteoporgenitor cells in the bone marrow and bone formation (mineralization surface, mineral apposition/bone formation rates) compared with vehicle group. In addition, medicarpin increased cortical thickness and bone biomechanical strength. In adult OVX mice, medicarpin at 10mg/kg dose protected trabecular bones against OVX-induced loss, and maintained trabecular microarchitecture (16). In adult OVX rats, medicarpin treatment resulted in the faster healing of femur osteotomy over the control by the likely participation of canonical Wnt and Notch pathways (19).

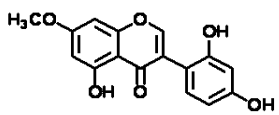
E2 deficiency is also known to upregulate TNF-α production from T cells by increasing the number of TNF-α-producing cells. Medicarpin prevents premature T cell senescence and bone loss via (a) increasing mRNA levels of nucleolin, hnRNP-D0A, and CD28 in BM T cells; (b) antagonizing TNF-α-induced loss of CD28 expression in an E2-dependent manner; and (c) abrogating TNF-α-induced ROS production (20). From these studies, it appears that medicarpin has osteoanabolic, anti-resorptive and immunomodulatory effects that support bone regeneration and protect against bone loss under the condition of E2 deficiency.

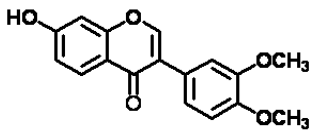
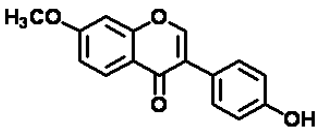
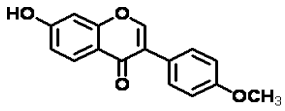
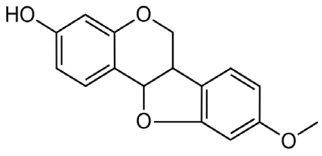
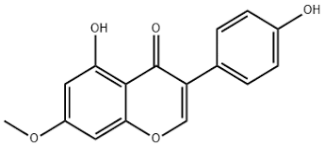
In adult rats the absolute bioavailability of medicarpin at 5- and 20 mg/kg doses were 5.2% and 2.6%, respectively. A 4-fold increase in dose did not result in equivalent increase in either C_{max} or AUC which could be due to solubility constraints at higher dose or due to precipitation of the medicarpin in intestinal lumen, thus, limiting the absorption. The poor oral exposure could also be due to first-pass effect. Medicarpin, containing a hydroxyphenyl and methoxyphenyl group may be prone to metabolism in liver and gut, via oxidation, demethylation or glucuronidation (2).

VIII. Summary

Table 1 shows the salient features of the bioactive compounds in *B. monosperma*'s stem-bark extract. From their in vitro effects, dependence on signaling pathways and in vivo effects, it appears that the extract through the synergistic and/or additive mode of action of these compounds has salutary effects in bone.

Table 1. Salient features of bioactive compounds from *B. monosperma*

Compounds	Osteogenic concentration	In vitro effects	Signaling pathways	In vivo effects	PK parameters	
					$AUC_{(0\text{-}24\ h)}$ ($ng.ml^{-1}.h^{-1}$)	Absolute bioavailability
Cajanin 	10^{-10}M	Proliferation & Differentiation	Sequential activation of MEK1/2 & Akt pathways	Increased bone strength, MAR & BFR	316.114 ± 23. 564	17.23%

Cladrin 	10^{-8}M	Proliferation & Differentiation	Activation of MEK1/2	Increased MAR & BFR	236.327 ± 37.25	13.7%
Isoformononetin 	10^{-8}M	Survival & Differentiation	Parallel activation of MEK1/2 & Akt pathways	Increased MAR & BFR	277.489 ± 33.698	10.46%
Formononetin 	10^{-7}M	Differentiation	Activation of p38 MAPK	No effect	147.248 ± 23.38	4.3%
Medicarpin 	10^{-10}M	Differentiation	Activation of estrogen receptor β ® bone morphogenetic protein-2 ® p38MAPK	Increased bone strength, MAR & BFR	65.40 ± 22.90	2.6%
Prunetin 	10^{-8}M	Proliferation & Differentiation	GPR30 cAMP ® MEK1/2	Bone regeneration at the fracture site	-	-

IX. References

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XI. Licensing status

Natural Remedies, Bengaluru.

B. Discovery and Pipeline Building

Chapter 5: Phytopharmaceutical assessment of Indian medicinal plants in bone disease

I. Introduction

We routinely carry out screening of Indian medicinal plants for their efficacy in various preclinical models of bone disease. We also carry out a rigorous phytochemical assessment of the plants with emphasis on discovery of novel compounds. Subsequently, we undertake screening of isolated compounds in bone cells including osteoblasts, osteoclasts, MSC, and chondrocytes. Active compounds are further pursued for understanding their modes of action, metabolic stability and in vivo efficacy in models of bone loss. Promising compounds in terms of drug-like properties are selected for chemical synthesis of series of compounds, the so called nature-inspired synthesis of novel molecules. Below are examples of medicinal plants that we have studied in details.

II. *Cupressus sempervirens*

The genus Cupressus (cupressaceae), comprising twelve species, is distributed in North America, the Mediterranean region and subtropical Asia at high altitudes. Five of them were reported as part of the Indian flora including *C. sempervirens*, *C. funebris*, *C. lusitanica* (*C. glauca*), *C. macrocarpa* and *C. cashmeriana*. Phytopreparation obtained from the core and young branches of *C. sempervirens* were reported to have antiseptic, aromatherapeutic, astringent, balsamic and anti-inflammatory activities.

The fruit of *C. sempervirens* contains two new phenolic glycosides including quercetin-3-*O*-(4″-methoxy)--L-rahmnopyranosyl and (2-hydroxy5-isopropyl-4-methyl-phenoxy)-4′-methoxy-β-D-glucopyranoside. Besides, there are fourteen known compounds including catechin, epicatechin, neolignans, 1-(4-hydroxy-3-methoxyphenyl)-2-[4-(3-rhamnopyranoxypropyl)-2-hydroxyphenoxy] 1,3-propanediol), (1-(4-hydroxyphenyl)-2-[4-(3- glucopyranoxypropyl)-2-methoxyphenoxy]-1,3-propanediol), sugiol, communic acid, junepediol, sandracopimaric acid, enantio olevaric acid, imbricatolic acid, acetoxyimbricatolic acid, ferruginol, and abita-8, 11, 13-triene-20-ol (1).

Sugiol, a diterpenoid promotes osteoblast differentiation at 1pM and increased bone volume and bone strength in OVX mice at 1mg/kg dose (2). Cupressuflavone, a bis flavone class of compound, in which the attachment of two apigenin units was through their corresponding C-8 and C-8 positions occurred and termed 8,8”-biapigeninyl (BA). BA was found to be 104-fold more potent than apigenin. The effect of BA in osteoblasts appeared to be mediated via estrogen receptors (ER) as antiestrogen, ICI-182780 abolished BA-stimulated osteoblast differentiation. In OVxmice BA treatment (at 1.0-, 5.0- and 10.0 mg/kg doses) given orally for 30 days dose-dependently inhibited mRNA levels of osteoclastic genes including tartrate-resistant acid phosphatase, receptor activator of nuclear factor (RANK), tumor necrosis factor alpha, interleukin-6 and the ratio of RANK ligand/osteoprotegerin

ratio in bones compared with OVx mice treated with vehicle. In addition, BA treatment to OVx mice dose-dependently stimulated production of osteoprogenitor cells in the bone marrow and increased mRNA levels of osteogenic genes core binding factor alpha-1, type I collagen and bone morphogenic protein-2 in bones compared with OVx + vehicle group. Microcomputed tomography revealed that BA treatment to OVx mice improved parameters of trabecular and cortical architecture. BA exhibited no uterine estrogenicity (3). From these data, we conclude that BA exerts osteoprotective effect in OVx mice by multiple beneficial effects on bone cells.

III. *Pterospermum acerifolium*

Pterospermum acerifolium (Linn.) Willd. belongs to the family Sterculiaceae is distributed through Southeast Asia, from India to Burma. The plant is locally known as Kanak Champa. *P. acerifolium* has a wide application in traditional system of Indian medicine. From the seed coat of this plant, 9 compounds were isolated out of which 4 are new. The new compounds include pteroceramide A, pteroceramide B, pterosterol A and pterosterol B. Other compounds were 5,7- dihydroxy-3-(4-hydroxy-3-methoxyphenyl)-6-methoxy-chromen4-one, β-sitosterol, lacinilene C, β-sitosterol-*O*-β-D-glucopyranoside and friedeline (4). Pteroceramide A and pteroceramide B were also present in the flower besides another new compound, pterospermin C (5). Pteroceramide A, pteroceramide B and pterospermin C significantly increased differentiation of rat calvarial osteoblasts (4, 5).

IV. *Ginkgo biloba*

A standardized extract of *Ginkgo biloba* (EGb 761) contains 24% flavonoids, 5–7% terpene lactones, 5–10% organic acids and other constituents. To adult OVX rats, 100mg/kg EGb treatment for 5 weeks resulted in higher BMD and lower bone turnover markers than OVX rats treated with vehicle. Kaempferol, quercetin and rutin are abundant constituents of flavonoid-rich EGb 761 and these compounds have been reported by others and us as osteoanabolic and anti-catabolic (6). Besides, EGb 761 enhances the commitment and differentiation of bone marrow stromal cells toward osteoblast lineage, whereas adipogenic differentiation and maturation (7).

We next studied the serum and bone marrow levels of kaempferol, quercetin and rutin to estimate their potential roles in carrying out the effect of Egb 761 in bone. Our data show that rutin has the least and quercetin has the highest bioavailability in serum and bone marrow of rats after Egb 761 treatment. In rats, kaempferol is known to be metabolized first to quercetin and then to rutin. In the case of Egb 761, we observed that although the amount of rutin is highest among the three flavonoids of Egb 761 and that metabolism of kaempferol and quercetin yield rutin, yet its low serum and bone marrow levels indicate that rutin has very poor bioavailability.

Attempt to correlate the levels of these flavonoids in the bone marrow with the beneficial outcomes on bone as observed in this study suggest involvement of kaempferol and quercetin, but not rutin in the process. Interestingly, kaempferol levels are comparable between serum and bone marrow whereas quercetin levels in the bone marrow was ~15% of its serum levels, indicating that

kaempferol is retained more efficiently than quercetin (6).

V. *Coelogyne cristata*

Coelogyne criststa (CC), family Orchidaceae, locally known as 'Hadjojen' (bone jointer) is commonly used for the treatment of fractured bones in folk traditions of India. Fresh plant material (leave and pseudo-bulb) of CC were subjected to ethanolic extraction followed by fractionation in hexane, ethyl acetate and methanol. Coelogin (7,8-dimethoxy-9,10-dihydro-5H-naptho [8,1,2-cde] chromene-2,6-diol] was isolated through fractionation. CC extract at doses of 5.0, 10.0 and 20.0 mg/kg body weight exhibited significant osteoprotective effects. Importantly, the extract was devoid of any uterine estrogenicity at any of these doses. The preservation of trabecular microarchitecture significantly adds to bone strength and may cut down fracture risk significantly. Additionally, CC extract treatment led to improved biomechanical properties as exhibited by increased stiffness, power and energy at all the three doses in femoral bones compared to untreated Ovx animals (8). Coelogin, the isolated compound from the extract promotes osteoblast differentiation likely via the estrogen receptors and favors osteoblast survival. In osteopenic mice, coelogin at 1mg/kg oral dose restored bone mass, microarchitecture and bone strength on a par with PTH (9). Overall, this study exhibits the potential of coelogin as a therapeutic agent in postmenopausal osteoporosis.

VI. *Passiflora foetida*

Passiflora foetida is an Indian medicinal plant belongs to the family passifloraceae is a herbaceous climber, native of tropical America and found in several parts of India. It is commonly called as stinking passion flower in English. The phyto-constituent of Passiflora foetida are flavonoids pachypodol, dimethoxyapigenin, ermanin, 7-*O*-dimethyl-naringenin, 3,5-dihydroxy-4,7-dimethoxy flavanone, *C*-glycosyl flavonoids chrysoeriol, apigenin, isovitexin, vitexin, xylosylvitexin, luteolin-7-β-D-glucoside, kaempferol, cyanohydrin glycosides tetraphyllin A, tetraphyllin B, tetraphyllin B sulphate, deidaclin, volkenin, fatty acids linoleic acid, linolenic acid and alpha-pyrones (passifloricins). Daily oral administration of phytopreparation (butanolic fraction) from *P. foetida* at 50- and 100mg/kg doses prevents OVx-induced bone loss in mice and therefore raises the chances of its use in postmenopausal osteoporosis (10).

VII. *Pholidota articulate*

Pholidota articulata Lindley (PA) belongs to family Orchidaceae and is distributed throughout montane to submontane zones from Uttarakhand Himalayas (Kumaon and Garhwal) to Aruunanchal Pradesh and Indo-China to Malaysia. Poultice made from PA locally known as Hadjojen (bone jointer), is one of the most common ailment used for healing fractures in folk tradition of Kumaon, Uttarakhand, India. In Ayurvedic formulation, it is referred as "Jivanti" and used as tonic.

The effect of an ethanol extract of PA was studied in a mouse of osteoporosis. At 5mg/kg oral dose, PA reversed the trabecular osteopenia and restored bone strength in OVX mice. Subsequently,

three phenanthrene derivatives viz flaccidin, flavidin, and oxoflavidin were isolated from ethyl acetate fraction of PA. In vitro osteogenic effect was observed in oxoflavidin which led to enhanced ALP activity (a marker of osteoblast differentiation), mineral nodule formation and mRNA levels of osteogenic markers like BMP-2, Type 1 Collagen, RUNX-2 and osteocalcin. These results validate the traditional claim of PA used for healing fractures. The identified bioactive compound may serve as the starting point for design and development of pharmaceutical products not only to reduce fracture risk but also for the management of postmenopausal osteoporosis (11).

VIII. Identification of osteogenic compounds from *Allophylus serratus, Cissus quadrangularis* and *Vitex negundo*

Total 14 compounds were isolated from the *n*-butanol fraction of the ethanolic extracts of these plants. Of these, rutin, 6'-*O*-trans-cinnamoyl-catalpol, agnuside, negundoside and luteolin showed in vitro osteogenic effect (12).

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Chapter 6: Outlook and future Directions

The Nobel Prize in medicine to Dr. Tu Youyou in 2015 for her discovery of artemisinin isolated from *Artemisia annua* for the treatment of malaria is the most recent recognition of the value of the plant-based drug discovery. On the heels of this celebration, a New Phytopharmaceutical Drugs program was notified by the Central Drugs Standard Control Organization (CDSCO) that permitted the development of the phytopharmaceutical drugs using advanced techniques such as solvent extraction, fractionation, potentiating steps, and modern formulation. After receiving approval of the New Drug Application (NDA) from CDSCO, the phytopharmaceutical agent would enjoy the same status of a new chemical entity (NCE)-based drug. The new phytopharmaceutical regulatory guidelines are in line with regulations in the USA, China, and other EU countries that are involved in the scientific evaluation and data generation of medicinal plants. This new regulation is expected to promote innovations and the development of new drugs from botanicals for unmet clinical needs through evidence-based routes that are acceptable to the present medical professionals from all disciplines. It is anticipated that this new regulation will facilitate phytopharmaceutical drug development from the academic and industry researchers separately as well as collaboratively.

CSIR-CDRI played a pioneering role in developing phytopharmaceutical drugs including standardized extracts of *Bacopa monnieri* (CDRI 08), *Picrorhiza kurroa* (Picroliv), *Commiphora mukul* (guggulipid), and *Dalbergia sissoo* for memory enhancement, liver health, lipid disorders, and bone health, respectively. Each of these extracts is defined by chemical markers and pharmacologically active ingredients along with pharmacognosy, preclinical efficacy, preclinical pharmacokinetics, and safety studies. Following these lines of preclinical development similar to that required by allopathic drugs allowed clinical assessments of these standardized phyto-extracts.

The research program detailed here not only moved CDRI's phytopharmaceutical research forward by feeding the drug pipeline but also opened new research avenues through natural compound-inspired new drug discovery. One such example is the discovery of a new chemical entity from medicarpin isolated from *Butea monosperma* that has led to the preclinical development of an oral fracture-healing compound. More such examples can be found in a separate document on the pharmaceutical research program of the ASTHI center.

Although standardization of an extract and its pharmacologic assessment involve phytochemicals in the form of small molecules, miRNAs and peptides present in plants are reported to be present in human blood. Rasayana, an important branch of Indian traditional medicine is considered to be a holistic therapy owing to diverse phytochemicals. The majority of the effects are attributed to polyphenols, terpenoids, and alkaloids which constitute between 1-3% of all constituents. Total protein contents of these plants vary from 2-6% that untapped sources of bioactive peptides/

proteins having a therapeutic effect. Besides, miRNAs that are global regulators of gene expression at both transcriptional and post-transcriptional levels resulting in pre-and post-transcriptional gene silencing (PTGS) are found in human circulation after oral consumption of plants. Several recent reports suggest therapeutic effects of plant-derived miRNAs. It is therefore reasonable to begin the study of the musculoskeletal effects of plant-derived peptides and miRNAs.

Another emerging area of research is the modulation of gut microbiota by the phyto-extracts which could unravel new mechanisms explaining their pharmacology. Besides, bone-specific metabolism of phytochemicals that are beginning to be recognized could shed new light on our understanding of their pharmacologic effect. Finally, phytochemicals are often considered pleiotropic in nature however we discovered GTDF from *Ulmus wallichiana* as a specific agonist of adiponectin receptor 1. Systematically performed targeted screening of the compound library derived from plants particularly for AdipoRs and glucagon-like peptide receptor 1 could lead to the discovery of the dual action bone and muscle anabolic compounds.