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A standardized extract of Danggui Buxue Tang decoction selectively exerts estrogenic activities distinctly from Tamoxifen

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1 **A standardized extract of Danggui Buxue Tang decoction selectively exerts**
2 **estrogenic activities distinctly from Tamoxifen**
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1

24 **Abstract**

25 More and more menopausal women use Danggui Buxue Tang (DBT) for relieving their

26 symptoms. Concerns for its safety have been raised as it contains phytoestrogen and

27 acts via estrogen receptors (ERs). Our study aimed to determine whether DBT could

28 selectively exert estrogenic activities and interact with tamoxifen in bone, brain, uterus

29 and breast by using ovariectomized (OVX) rats and ER-positive cells. In OVX rats,

30 DBT induced a 31.4% increase in bone mineral density and restored the mRNA

31 expression of dopamine biomarker in striatum, 3.32-fold for tyrosine hydrolase

32 ($p<0.001$) and 0.21-fold for dopamine transporter ($p<0.001$), which was similar to

33 tamoxifen; tamoxifen, but not DBT, increased uterus weight and Complement

34 component 3 expression by more than two fold ($p<0.001$); unlike tamoxifen, DBT

35 induced mild proliferation in mammary gland. Two-way ANOVA indicated the

36 interactions between them in OVX rats ($p<0.05$) but DBT did not alter the responses to

37 tamoxifen. DBT stimulated proliferation or differentiation and estrogen response

38 element in MCF-7, MG-63, Ishikawa and SHSY5Y cells and altered the effects of

39 tamoxifen. In summary, DBT exerted estrogenic effects in tissue-selective manner,

40 which was different from tamoxifen. DBT interacted with tamoxifen but did not

41 significantly alter its effects in OVX rats.

42 *Keywords*: Danggui Buxue Tang decoction; phytoestrogen; estrogenic activities;

43 estrogen receptors; selective estrogen receptor modulators (SERMs); tissue-selectivity

44

45 *Abbreviation:*

46 ALP, alkaline phosphatase; AP-1, Activator Protein-1; BMD, Bone mineral density;

47 CAMs, complementary and alternative medicines; DBT, Danggui Buxue Tang; ER,

48 Estrogen Receptors; ERE, Estrogen Response Element; HPG, Hypothalamus-pituitary-

49 gonadal; HRT, Hormone Replacement Therapy; OVX, Ovariectomy; RA, *Radix*

50 *Astragali*; RAS, *Radix Angelicae Sinensis*; SERMs, Selective Estrogen Receptor

51 Modulators; TCMs, Traditional Chinese medicine.

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1   INTRODUCTION

53   Hormone replacement therapy (HRT) with the use of exogenous estrogen alone or in
54   combination with progestin has been regarded as the gold standard for management of
55   menopausal symptoms. Indeed, HRT carries considerable benefits for treatment of
56   vasomotor symptoms and osteoporosis, but the increased risks of reproductive cancers,
57   stroke and cardiovascular diseases make HRT a subject of debate (Miller *et al.*, 2017).
58   As an alternative approach to HRT, selective estrogen receptor modulators (SERMs)
59   are prescribed for postmenopausal women. Tamoxifen is an antagonist of estrogen
60   receptor (ER) present in breast tissue and currently utilized for treatment of ER-positive
61   breast cancer. Besides, tamoxifen is an ER agonist in bone tissue and has also been used
62   for prevention of menopausal osteoporosis (Maximov *et al.,* 2013). However,
63   tamoxifen not only induces depression, hot flushes and uterine abnormalities, but also
64   dramatically increases the risk of endometrial cancer. Consequently, menopausal
65   women who are unwilling to continue HRT nor to use SERMs would turn to the use of
66   complementary and alternative medicines (CAMs) (Cardini *et al.*, 2010) that would
67   yield comparable benefits as hormone therapy but with no or fewer inconvenience or
68   risk (Peng *et al.,* 2014). Phytoestrogens derived from plants are the most popular
69   alternative approach among CAMs (Moreira *et al.,* 2014). Phytoestrogens have been
70   demonstrated to activate ERs and exhibit various estrogenic and anti-estrogenic effects
71   in the same way as SERMs. As a main source of phytoestrogens, herbal medicines are
72   vigorously promoted because of their effectiveness and fewer side effects (Moreira *et*
73   *al.*, 2014). Indeed, demands for herbal medicine for management of menopausal
74   symptoms are increasing globally.

75   Among the herbal formulae used for relieving menopausal symptoms, Danggui
76   Buxue Tang (DBT) decoction is a simple combination of two herbs, 10 qian (an ancient
77   weight unit, 1 qian equals about 3 g) of *Radix Astragali* (RA, Huangqi) and 2 qian of
78   *Radix Angelicae Sinensis* (RAS, Danggui). It was first described by Li Dongyuan in
79   *Neiwaishang Bianhuo Lun* in AD 1247 and has been commonly prescribed for Chinese
80   women as a remedy for menopausal symptoms (Haines *et al.,* 2008; Wang *et al.,* 2013;
81   Zheng *et al.,* 2012). DBT effectively alleviated vasomotor symptoms in Hong Kong
82   Chinese postmenopausal women (Haines *et al*., 2008) and follow-up study further
83   demonstrated that the effectiveness of DBT even lasted until one month after drug
84   withdrawal (Wang *et al*., 2013). Our earlier studies reported a standardized DBT extract
85   and analyzed the chemical components of this extract. Among the four main
86   components, three (calycosin, formononetin, and ferulic acid) of them are flavonone
87   phytoestrogens (Choi *et al.,* 2011; Dong *et al.,* 2006; Gao *et al.,* 2007). DBT has been
88   shown to mimic estrogen effects and significantly increase the activities of estrogen
89   response element (ERE) in human osteosarcoma MG-63 cells (Choi *et al*., 2011; Zhou

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91 *et al.,* 2018). In particular, the effects of DBT was shown to be ER-dependent (Gao *et
92 al.*, 2007). In preclinical animal model, three month treatment with DBT dramatically
93 attenuated estrogen deficiency-induced bone loss in mature ovariectomized (OVX) rats,
94 confirming the estrogen-like anabolic effects of DBT in bone tissue (Zhou *et al.*, 2018).
95 Moreover, the bone beneficial activity of DBT was demonstrated to be associated with
96 its regulation on circulating estradiol and follicle stimulating hormone (FSH) level in
97 OVX rats. These results suggest that DBT contains phytoestrogens that exert estrogen-
98 like activities via ERs.

99   With the increasing popularity of using herbal medicine for treatment of
100 menopausal symptoms, concern has been raised for their safety, especially those
101 containing phytoestrogens. As phytoestrogens act via the same ERs, they might carry
102 similar risk-benefit profile as estrogen and SERMs. Therefore, it is of particular
103 importance to investigate whether these phytoestrogen-containing herbal medicine, like
104 DBT, could selectively exert beneficial effects in target tissues without inducing
105 undesirable actions in reproductive tissues. Moreover, it will be crucial to determine if
106 DBT interacts with tamoxifen to either increase of decrease the pharmacological
107 activities of tamoxifen, which is of particular concern to the breast cancer patients who
108 seek to take supplements together with their standard treatment to prevent recurrence
109 or to treat their menopausal symptoms. Therefore, the present study aimed to
110 characterize the estrogenic or anti-estrogenic effects of DBT in comparison to
111 tamoxifen as well as their interactions in four estrogen sensitive tissues, including bone,
112 brain, breast and uterus, by using *in vivo* mature OVX rats and *in vitro* ER-positive cell
113 lines.

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115 **2   METHODS**

116

117 **2.1 Preparation and chemical analysis of Danggui Buxue Tang (DBT) extract**
118 The standardized DBT extract used in the present study was prepared as described in
119 our previous studies (Choi *et al.*, 2011; Zhou *et al.*, 2018). Briefly, fresh roots 3-year-
120 old *A. membranaceus var. mongholicus* and 2-year-old *A. sinensis* were purchased from
121 Shanxi and Minxian of Gansu, China, respectively. 250 g of RA and 50 g of RAS were
122 mixed exactly at the ratio of 5:1 and boiled 8 volumes of water (v/w) for 2 hrs and
123 extracted twice following the ancient recipe that had been proven to have the best
124 extraction conditions (Song *et al.*, 2004). The extracts were dried by lyophilization and
125 stored -80 ℃. Contents of the chemical markers of standardized DBT extract were
126 shown in supplementary table 1.

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128 **2.2 Animal experiment and sample measurements**

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129   The animal experiment protocol conducted was approved by the Hong Kong
130   Polytechnic University Animal Subjects Ethics Sub-committee (ASESC Case: 12/11).
131   Six-month-old female Sprague Dawley rats (280-300 g) were purchased from the
132   Chinese University of Hong Kong. Animals were given bilateral ovariectomy or sham-
133   operated under anesthesia of ketamine (50 mg/kg) and xylazine (10 mg/kg). After two
134   weeks recovery, OVX-operated rats were randomly divided into treatment with
135   distilled water (OVX), water suspension of 17ß-estradiol (E8875, Sigma; E2, 2.0
136   mg/kg.day;), water suspension of tamoxifen (T5648, Sigma; Tamo-L, 0.1 mg/kg.day
137   and Tamo-H, 1.0 mg/kg.day), water solution of DBT (3.0 g/kg.day) or the combinations
138   of DBT and tamoxifen for 3 months and paired fed with phytoestrogen-free AIN-93M
139   diet (supplementary table 2) during the whole treatment. Sham-operated rats were
140   employed as blank control (sham). The dosage of E2, tamoxifen and DBT were selected
141   based on its clinical dose and our previous studies (Zhou *et al.*, 2018; Goetz *et al.*, 2017).
142   30 g of RA and 6 g of RAS has been prescribed for postmenopausal women with 60-
143   80 kg in body weight. The human dose should be 0.45-0.6 g/kg, which is converted into
144   rat dose as 2.79-3.72 g/kg by multiplying the conversion factor "6.2". And DBT at 3
145   g/kg has been demonstrated to be effective in prevention of postmenopausal
146   osteoporosis in ovariectomized rats in our previous study (Zhou *et al.*, 2018). Upon
147   treatment, serum, uterus, breast tissue, striatum, lumbar spine, and tibial head were
148   collected. Serum estradiol level was measured by EIA kit (Cayman, US). mRNA
149   expression of estrogen responsive genes in uterus, striatum and tibial head were
150   measured by real-time quantitative reverse transcriptase-polymerase chain reaction
151   (PCR) assay. Bone properties of lumbar spine was measured by micro-CT analysis as
152   previously described (Zhou *et al.*, 2018). Hematoxylin-Eosin (H&E) staining was
153   performed for the pathohistological analysis of uterus and mammary gland.

154

155   **2.3 BMD and Micro-CT analysis**

156   Bone properties of trabecular bone at proximal tibia and distal femur as well as lumbar
157   vertebra were determined by Micro-CT (μCT40, Scanco Medical, Switzerland). The
158   source energy selected for this study was 70 KVp and 114 μA with resolution of 21 μm.
159   Approximately 200 slices were done for each scan. The distal/proximal were defined
160   as 4.2 mm and 2.2 mm away from femur/tibia head. Scanning was done at the
161   metaphyseal area located 0.63 mm below the lowest point of the epiphyseal growth
162   plate and extending 2.0 mm in the proximal direction. Bone mineral density (BMD, mg
163   HA/ccm) and bone morphometric properties, including bone volume over total volume
164   (BV/TV), connectivity density (Conn.D, $1/mm^3$), structure model index (SMI),
165   trabecular bone number (Tb.N, mm^{-1}), trabecular bone thickness (Tb.Th, mm) and

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166 trabecular bone separation (Tb.Sp, mm), were evaluated by contoured volume of
167 interest (VOI) images.

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169 **2.4 Hematoxylin-Eosin (H&E) staining**

170 Collected tissues were fixed in 4% paraformaldehyde for 6 hrs. Upon dehydration
171 (Leica TP1020), tissues were embedded in paraffin and 8 μm-thick sections were
172 produced for each sample. At minimum of 5 sections from each sample were observed
173 using 100× or 400× magnification and photographed using a photoscope (Olympus
174 BX51).

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176 **2.5 Cell culture and measurement**

177 Human breast MCF-7 cells (ATCC® HTB-22™, passage 8-15), endometrial Ishikawa
178 cells (kindly provided by Dr. Lihui Wei at Peking University People's Hospital, passage
179 12-18), neuroblastoma SHSY5Y cells (ATCC®CRL-2266™, kindly provided by Prof.
180 Wenfang Chen at Qingdao University, passage 10-20) and osteosarcoma MG-63 cell
181 (ATCC® CRL-1427™, Passage 3-10) were routinely cultured according to ATCC
182 instruction (as shown in supplementary table 3). Cells were seeded in 96-well or 24-
183 well plate at a density of 0.8×10^3 and 2.0×10^4 /well, respectively, for different assays.
184 The medium was changed to phenol red-free (PRF) medium containing charcoal-
185 stripped FBS (cs-FBS) for another 24 hrs. Cells were treated with DBT at various
186 concentrations (0.05, 0.1, 0.25, 0.5, 1.0 and 2.0 mg/ml), tamoxifen (10^{-12} to 10^{-6}M) and
187 their combinations at optimal concentrations for 48 hrs. Cell viability or ALP activity
188 were measured by MTS assay or ALP assay, respectively. Cells were transfected with
189 0.4 μg of ERETkluc plasmid and estrogen response element (ERE)-luciferase activity
190 was measured as previously described (Zhou *et al.*, 2018).

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192 **2.6 Real-time PCR assay**

193 Collected tissues were homogenized in Trizol reagent by using Precellys 24
194 homogenizer (Bertin, France). Total RNA, reverse transcription and quantitative PCR
195 were carried out as previously described (Zhou *et al.*, 2018). Briefly, 2.0 μg of total
196 RNA was reverse-transcribed into cDNA by using High-Capacity cDNA Reverse
197 Transcription Kits (Applied Biosystem) following the manufacturers' instruction. 20 μl
198 of PCR reaction system consisting of 1 μl cDNA, 0.4 μl of forward and reverse primers,
199 8.2 μl of DNase and RNase-free water and 10 μl of SsoFast™ EvaGreen® Supermix
200 (Bio-Rad) was performed by using Iq5 Multicolor Real-time PCR Detection System
201 (Bio-Rad, IQ5). Sequences and conditions for primers were provided in supplementary
202 table 4.

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204 **2.7 Statistical analysis**

205 Data was reported as mean ± SEM. Inter-group differences of *in vivo* study were

206 analyzed by One-way ANOVA with Tukey post hoc test. Differences between

207 treatment group and control of *in vitro* study were determined by Independent t-test.

208 Herb-drug interactions were analyzed by Two-way ANOVA with Bonferroni as post

209 hoc test. A *p* value < 0.05 was considered statistically significant.

210

211 **3 RESULTS**

212

213 **3.1 Estrogenic activities of DBT and its combinations with tamoxifen in mature**

214 **ovariectomized (OVX) rats**

215 Body weight significantly increased in OVX rats (vs. sham rats, *p*<0.001) and was

216 reversed in OVX rats by treatment with E2 and tamoxifen (*p*<0.001), but not DBT (Fig.

217 1A). Co-treatment with DBT significantly attenuated the inhibitory effects of tamoxifen

218 on body weight gain at both low and high dose (*p*<0.05). Results of Two-way ANOVA

219 indicated the interaction between DBT and tamoxifen at low dose, but not tamoxifen at

220 high dose, to decrease the inhibitory effect of tamoxifen on body weight gain (DBT ×

221 Tamo-L: *p*=0.0001). The decline in serum estradiol levels in rats upon OVX operation

222 was completely restored by E2 supplementation to the level higher than that in sham

223 rats (*p*<0.001) (Fig. 1B). Tamoxifen alone, DBT alone and their combinations appeared

224 to increase the estradiol level in OVX rats while the changes did not reach statistical

225 significance. In particular, estradiol level in OVX rats treated with DBT was

226 comparable level to that in sham rats (33.47±4.73 pg/ml in sham rat vs 43.35±4.32

227 pg/ml in DBT-treated rats). According to Two-way ANOVA, there was no interaction

228 between DBT and tamoxifen on stimulating estradiol level.

229 Uterine weight of OVX rat was significantly reduced to about 25% of sham rats

230 (Fig. 2A, vs. sham rats, *p*<0.001) and an obvious atrophy was observed in the uterus of

231 OVX rats as revealed by the shrunken endometrium (Fig. 2C). Treatment with E2,

232 tamoxifen alone or its combinations with DBT, but not DBT alone, significantly

233 increased the uterus weight in OVX rats (*p*<0.01), confirming the uterotrophic effects

234 of tamoxifen. The changes in uterus weight by different treatments were in consistence

235 with the increase in thickness of endometrium (Fig. 2C). Similar to estradiol, tamoxifen

236 alone at both doses and combinations with DBT, but not DBT alone, appeared to

237 increase the mRNA expression of *complement component 3* (*C3*), an estrogen

238 responsive gene, in uterus of OVX rats. Results of Two-way ANOVA suggested that

239 DBT interacted with tamoxifen at high dose on uterus weight of OVX rats (DBT ×

240 Tamo-H: *p*=0.0012). Ovariectomy also induced atrophy of breast tissue, as indicated

241 by the reduced number of mammary ducts in OVX rats; estrogen increased the size and

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number of mammary duct while tamoxifen enhanced the atrophy in mammary gland of OVX rats (Fig. 3). DBT was observed to induce a mild increase in the number of mammary ducts, indicating the potential estrogenic activity of DBT in breast tissue. These results indicated that DBT did not induce estrogenic effects in uterus but slightly stimulated mammary gland in OVX rats, and such actions of DBT were completely opposite to those of tamoxifen.

As expected, estrogen deficiency induced significant bone loss in OVX rats as revealed by the deteriorated bone structure (Fig. 4A) and the decrease in BMD at lumbar vertebra (Fig. 4B, $p<0.001$). Tamoxifen alone, DBT alone and their combinations significantly restored OVX-induced changes in bone structure and BMD ($p<0.001$). OVX up-regulated the mRNA expression of *Interleukin-6* (*IL-6*) and down-regulated *alkaline phosphatase* (*ALP*) (markers for bone resorption and bone formation, respectively) in tibial head of rats, indicating the disturbance of bone remodeling. Treatment with tamoxifen alone, DBT alone and their combinations significantly suppressed the *IL-6* mRNA expression in tibial head to comparable levels to that of sham rats, suggesting their inhibitory effects on bone resorption (Fig. 4C, $p<0.05$). On the other hand, DBT and its combination with tamoxifen, but not tamoxifen alone, significantly restored *ALP* mRNA level in tibial head of OVX rats (Fig. 4D, $p<0.05$). Two-way ANOVA suggested that DBT interacted with tamoxifen at high dose on increasing BMD at lumbar spine (DBT × Tamo-H: $p=0.0058$) and interacted with tamoxifen at both doses to suppress *IL-6* mRNA expression in tibial head (DBT × Tamo-L: $p=0.0004$; DBT x Tamo-H: $p=0.0026$).

To determine if DBT also exerted estrogenic activities in central nervous system, its effects on mRNA expressions of *tyrosine hydroxylase* (Latourelle *et al.,*) and *dopamine transporter (DAT)* in striatum were determined. The mRNA expression of *TH* was reduced while the mRNA expression of *DAT* was significantly increased in striatum in OVX rats (Fig. 5, $p<0.001$). Treatment with E2, tamoxifen alone, DBT alone or in combination with tamoxifen significantly restored OVX-induced changes in *TH* and *DAT* mRNA expression in striatum of OVX rats ($p<0.05$). These results indicated the potential beneficial actions of DBT in central nervous system. Two-way ANOVA suggested that DBT interacted with tamoxifen to suppress *DAT* mRNA expression in striatum of OVX rats (DBT × Tamo-L: $p=0.0075$; DBT × Tamo-H: $p=0.0060$).

3.2 Direct estrogenic activities of DBT and combination with tamoxifen *in vitro*

The direct estrogenic effects of DBT were evaluated in human breast cancer MCF-7 cells, endometrial cancer Ishikawa cells, neuroblastoma SHSY5Y cells as well as osteosarcoma MG-63 cells, in line with the four estrogen sensitive tissues characterized in the OVX rats. DBT significantly exerted stimulatory effects on cell viability of MCF-

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7 and SHSY5Y cells as well as on ALP activity of Ishikawa and MG-63 cells (data not shown). Optimal concentrations of DBT were used in following experiment. As expected, tamoxifen remarkably inhibited cell viability and induced ERE activities in MCF-7 cells. In contrast, DBT at 0.1 and 0.5 mg/ml significantly stimulated cell viability as well as ERE-dependent transcriptional activities in MCF-7 cells (Fig. 6A, 7A). DBT at 0.1 and 0.5 mg/ml promoted ERE activity by 1.7 and 3.6-fold, respectively. Two-way ANOVA indicated that DBT at 0.5 mg/ml interacted with tamoxifen at 10^{-12} M ($p=0.0122$), 10^{-10} M ($p=0.0004$) and 10^{-8} M ($p=0.0032$) and significantly reversed the inhibitory effects of tamoxifen on cell viability in MCF-7 cells (Fig. 7A, $p<0.001$). Furthermore, DBT at 0.1 mg/ml also reversed the inhibitory effects of tamoxifen on cell viability in MCF-7 cells ($p<0.001$).

Tamoxifen did not alter ALP activities and ERE-dependent transcriptional activities in Ishikawa cells. In contrast, DBT at 0.5 and 1 mg/ml stimulated ALP activities as well as ERE-dependent transcriptional activities in Ishikawa cells (Fig. 6B, 7B). ERE activity was increased by 0.35 and 0.46-fold upon treatment with DBT at 0.5 and 1.0 mg/ml, respectively (vs. control, Fig. 6B). According to Two-way ANOVA analysis, DBT at 0.5 mg/ml interacted with tamoxifen at 10^{-6} M (Fig. 7B, $p=0.0025$), but did not alter the effect of tamoxifen on ALP activity. Co-treatment with DBT at 1.0 mg/ml significantly altered the effect of tamoxifen at lower concentrations (10^{-12} M, 10^{-10} M and 10^{-8} M) on ALP activity (Fig. 7B).

Tamoxifen stimulated cell viability from 10^{-12} to 10^{-8} M but did not activate ERE-dependent transcriptional activities in SHSY5Y cells (Fig. 6C, 7C). In contrast, DBT at 0.25 and 0.5 mg/ml significantly increased cell viability and ERE-dependent activities in SHSY5Y cells (Fig. 6C, 7C). Upon treatment with DBT at 0.25 and 0.5 mg/ml, ERE activity in SHSY5Y cells was increased to 1.3 and 1.2-fold to that of control, respectively. Two-way ANOVA indicated that DBT interacted with tamoxifen at all the doses applied (DBT at 0.25 mg/ml, $p=0.0094$ for tamoxifen at 10^{-12} M, $p=0.0002$ for tamoxifen at 10^{-10} M, $p=0.0025$ for tamoxifen at 10^{-8} M, $p=0.0003$ for tamoxifen at 10^{-6} M; DBT at 0.5 mg/ml, $p=0.0052$ for tamoxifen at 10^{-12} M, $p=0.0151$ for tamoxifen at 10^{-10} M, $p=0.0141$ for tamoxifen at 10^{-8} M, $p=0.0294$ for tamoxifen at 10^{-6} M) and significantly enhanced the stimulatory effects of tamoxifen on cell viability in SHSY5Y cells.

Tamoxifen did not alter ALP activities at 10^{-12} to 10^{-6} M but significantly induced ERE-dependent transcriptional activities at 10^{-7} and 10^{-6} M in MG-63 cells ($p< 0.01$, Fig. 6D, 7D). DBT at 1.0 and 2.0 mg/ml significantly increased ALP activities and ERE-dependent transcriptional activities in MG-63 cells ($p<0.001$, Fig. 6D, 7D). In particular, the effect of DBT at 2.0 mg/ml on ERE activity was much more potent than that of estradiol ($p<0.001$ vs E2). Two-way ANOVA indicated that DBT interacted

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with tamoxifen and significantly enhanced the action to increase ALP activity in MG-63 cells (DBT at 1.0 mg/ml, p=0.0055 for tamoxifen at 10^{-12} M, p<0.0001 for tamoxifen at 10^{-10} M, p=0.0003 for tamoxifen at 10^{-8} M, p=0.0134 for tamoxifen at 10^{-6} M; DBT at 2.0 mg/ml, p<0.0001 for tamoxifen at 10^{-12} M, p<0.0001 for tamoxifen at 10^{-10} M, p<0.0001 for tamoxifen at 10^{-8} M).

4 DISCUSSION

Our previous studies demonstrated that DBT contained phytoestrogens and exerts bone protective effects possibly via modulating the hypothalamus-pituitary-gonadal (HPG) axis (Zhou *et al.*, 2018). The present study further demonstrated that DBT exerted selective estrogenic effects in mature OVX rats as well as ERE-dependent estrogen-like activities in ER-positive cells. *In vivo* study demonstrated the herb-drug interactions between DBT and tamoxifen at their respective clinical dosages but DBT did not significantly alter the tissue responses to tamoxifen. *In vitro* study also reported the interactions between DBT and tamoxifen, and DBT was shown to significantly alter the effects of tamoxifen at certain concentrations in ER-positive cells.

The effects of DBT in breast and uterus were different from those of estrogen and tamoxifen. Tamoxifen acts as ER antagonist in breast and clinically is prescribed for treatment of ER-positive breast cancer (Maximov *et al.*, 2013). In our study, tamoxifen enhanced OVX-induced atrophy in mammary gland. In uterus, tamoxifen is an ER agonist and has been reported to clinically increased risk of endometrial cancer (Maximov *et al.*, 2013). This is in line with our observation that tamoxifen increased uterus weight and thickened endometrium of OVX rats. It is surprising that DBT only induced mild estrogenic effect in mammary gland and did not affect uterus in OVX rats, which showed an increased circulating estradiol level upon treatment with DBT. In fact, it is the local estradiol, rather than the circulating estradiol, that determines the estrogenic effects of estradiol in local tissues (Huhtinen *et al.,* 2012). Phytoestrogens have been reported to facilitate the clearance of estrogens from local tissues like uterus and breast and catabolize the metabolism of estrogens (Wood *et al.,* 2007), decreasing the local estradiol level and regulating responses in local tissues as observed in our study. The discrepancy in results between *in vitro* (Ishikawa and MCF-7 cells) and OVX rats might be due to the differences in concentrations of DBT used *in vivo* and *in vitro* as the concentrations of DBT applied to cells were too high to be reached *in vivo*. Another possible explanation is that DBT used in cells was not biologically activated. Therefore, the *in vivo* results should be more relevant that DBT selectively exerted mild estrogenic activity in breast, but not uterus, which was different from tamoxifen.

Tamoxifen was shown to exert comparable bone protective effect as estrogen in prevention of OVX-induced bone loss in our study, confirming that tamoxifen is ER

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356　agonist in bone (Maximov *et al.*, 2013). Compared to tamoxifen, the bone protective

357　activity of DBT was slightly weaker as decrease in BMD was only partially restored.

358　Similarly to estrogen and tamoxifen, DBT suppressed the mRNA expression of *IL-6*

359　(an estrogen sensitive bone resorption marker) in tibial head (Clowes *et al.,* 2005) while

360　DBT, not estrogen or tamoxifen, significantly promoted *ALP* mRNA expression in

361　tibial head. These findings suggest that DBT protects bone via both suppressing bone

362　resorption and stimulating bone formation while tamoxifen mainly suppresses bone

363　resorption (Morita *et al.*, 2016). Epidemiological studies suggested that

364　postmenopausal women have increased risk of Parkinson's disease (Erickson *et al.,*

365　2007). Indeed, our results showed that OVX induced dramatic decrease in tyrosine

366　hydroxylase (*TH*) and increase in dopamine transporter (*DAT*) mRNA expression in

367　striatum of OVX rats, indicating the interrupted dopamine metabolism in estrogen

368　deficient condition (Latourelle *et al.*, 2010), which were undoubtedly restored by

369　estrogen. Tamoxifen also restored changes in *TH* and *DAT* mRNA expression in OVX

370　rats and stimulated cell viability in neuron cells in ERE-dependent manner, suggesting

371　the potential neuroprotective effects, especially in estrogen deficiency conditions. Such

372　results were in line with those studies by others in which tamoxifen exerted

373　neuroprotective effects *in vivo* and *in vitro* (Lee *et al.,* 2009; Mosquera *et al.,* 2014).

374　DBT exerted comparable neuroprotective effects in striatum to those of estrogen and

375　evidently promoted neuronal SHSY5Y cells. In addition to increasing estradiol level,

376　DBT was shown to induce nerve growth factor (NGF) and brain-derived neurotrophic

377　factor (BDNF) in SHSY5Y cells (Gong *et al.,* 2017), which might also be involved in

378　mediating the neuroprotective activity of DBT. Moreover, the restoration of DBT on

379　dopamine metabolism might explain the improved mental health of menopausal women

380　using DBT as dopamine is the happiness hormone (Baixauli Gallego, 2017). Most

381　importantly, these findings clearly suggest that DBT reduces the risks of menopause

382　related skeletal and neurological disorders in a manner similar to tamoxifen.

383　　　　The estrogenic activity in local tissues are mediated by ERs and the relative tissue

384　expression of ER subtypes (ie, ERα and ERß) are important determinants of the tissue

385　response (Riggs and Hartmann, 2003). Tamoxifen has similar affinity for the ERs as

386　estrogen (Cronin-Fenton *et al.*, 2014) and shows equivalent affinity for both subtypes

387　of ER (Heldring *et al.,* 2004), resulting in the antagonistic action in breast and agonistic

388　action in uterus (Martinkovich *et al.,* 2014; Maximov *et al.*, 2013). The subsequent

389　discovery that tamoxifen acts as ER agonist in bone widens its application as a

390　prevention of osteoporosis (An *et al.*, 2016). Bone, breast and uterus express more ERα

391　than ERß, which may explain our observations in OVX rats (Nilsson and Gustafsson,

392　2011; Powell *et al.*, 2008). Besides, beneficial actions against OVX-induced damages

393　in dopamine metabolism were observed in our study, indicating a potential ER agonistic

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394   activity of tamoxifen in nervous system, which needs further investigation. DBT is a

395   mixture of phytoestrogens with mixed estrogenic and antiestrogenic properties via

396   selectively binding to ERα or ERß (Zingue *et al.*, 2017a; Zingue *et al.*, 2019). As one

397   of the main active components of DBT, Calycosin has been demonstrated to

398   predominantly mediates its estrogenic activities and exhibits much weaker affinity but

399   significant antagonistic activities to ERα and ERß against estrogen (Gong *et al.*, 2017;

400   Tang *et al.,* 2010). Moreover, the estrogenic or antiestrogenic activity of phytoestrogen

401   was demonstrated to depend on their local concentrations as some of them exerted

402   antiestrogenic activity at higher concentrations but some at lower concentrations

403   (Zingue *et al.*, 2017b; Zingue *et al.*, 2019). Structure has been demonstrated to have

404   great impact on the activity and selectivity of phytoestrogen towards ERs in different

405   mammalian system (Djiogue *et al.*, 2010). These finding might explain our observation

406   that DBT exhibited estrogen-like activities in bone and brain but antiestrogenic activity

407   in uterus. Although the distinct affinity and preference for ERα and ERß as well as the

408   concentration-dependent estrogenic or antiestrogenic properties might provide insights

409   into the understanding of the discrepancy between DBT and tamoxifen, further study

410   on the underlying mechanism is still needed. As they both target ERs, it is of special

411   importance to see if phytoestrogens will interact with tamoxifen to alter the estrogenic

412   actions of each other, especially for those postmenopausal women who are taking

413   tamoxifen and phytoestrogens or phytoestrogens-containing herbal products, such as

414   DBT, for relief of menopausal symptoms. Our observations that DBT interacted with

415   tamoxifen but did not alter the beneficial effects of tamoxifen in bone, brain and breast

416   nor worsen its side effects in uterus of OVX rats might be explained by the preferred

417   binding of ERs to tamoxifen, but not phytoestrogens in DBT, due to the saturation of

418   ERs by more potent ligands (Cronin-Fenton *et al.*, 2014; Zingue *et al.*, 2019).

419   Above all, DBT selectively exerts estrogenic activities in OVX rats in different

420   manner from tamoxifen and interacts with tamoxifen without altering the tissue

421   responses to tamoxifen, suggesting DBT alone and in combination with tamoxifen

422   might be effective and safe alternative approaches for the management of menopause

423   related symptoms. However, a major limitation of the present study was that only a

424   single dose of DBT (clinical equivalence dose) was used alone and in combination with

425   two doses of tamoxifen in the *in vivo* study. Future study will be needed to confirm the

426   efficacy and safety for the use of DBT in combination with tamoxifen in human as

427   complementary approach for management of postmenopausal symptoms.

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429

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CONFLICT OF INTEREST

436   The authors have no conflicts of interest to disclose.

437

AUTHOR CONTRIBUTIONS

439   Liping Zhou performed the experiments, sample detection, data analysis and wrote the
440   manuscript. Ka-Ying Wong, Sisi Cao, Christina Chui-Wa Poon, Wenxuan Yu, and
441   Xiaoli Dong helped with the performing and sample collection of the animal experiment.
442   Karl Wah-Keung Tsim provided the DBT extract and chemical analysis. Man-Sau
443   Wong conceived and supervised the experiments and finalized the manuscript. All
444   authors reviewed the manuscript.

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601   **Figure legend**

602   **Figure 1 Estrogenic effects of DBT, tamoxifen and their combinations on body**

603   **weight gain and circulating estradiol level in mature ovariectomized rats**   Six-

604   month-old mature Sprague Dawley sham-operated (Sham) or ovariectomized (OVX)

605   rats were treated with either vehicle, 17ß-estradiol (2.0 mg/kg.day), tamoxifen (Tamo-

606   L, 0.1 mg/kg.day and Tamo-H, 1.0 mg/kg.day), DBT (3.0 g/kg.day) and combinations

607   of DBT and tamoxifen for 12 weeks. **A**. Body weight gain to the baseline were

608   compared between groups at six time points; **B**. Circulating level of estradiol was

609   measured by using EIA kit (CayMan). Data was expressed as mean ± SEM. n=6 to 12.

610   ***$p<0.001$ vs sham; ^^^$p<0.001$ vs OVX; #$p<0.05$, ###$p<0.001$ vs tamoxifen alone.

611

612   **Figure 2 Estrogenic effects of DBT, tamoxifen and their combinations on uterus**

613   **index, mRNA expression of estrogen responsive gene and endometrial morphology**

614   **in mature ovariectomized rats** Six-month-old mature Sprague Dawley sham-operated

615   (Sham) or ovariectomized (OVX) rats were treated with either vehicle, 17ß-estradiol

616   (2.0 mg/kg.day), tamoxifen (Tamo-L, 0.1 mg/kg.day and Tamo-H, 1.0 mg/kg.day),

617   DBT (3.0 g/kg.day) and combinations of DBT and tamoxifen for 12 weeks. Uterus was

618   collected and weighed upon sacrifice. Ratio of uterus weight to body weight was

619   recorded as uterus index (mg/g) and compared between groups (**A**). mRNA expression

620   of *complement component 3 (C3)* was measured by real-time PCR (**B**). Morphology of

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621 endometrium (400X, thickness of endometrium was indicated by the length of the red
622 line) was visualized by H&E staining (**C**). Data was expressed as mean ± SEM. n=5 to
623 12. ***$p<0.001$ vs sham; ^$p<0.05$, ^^$p<0.01$, ^^^$p<0.001$ vs OVX.

625 **Figure 3 Estrogenic effects of DBT, tamoxifen and their combinations on**
626 **morphology of breast tissue in mature ovariectomized rats** Six-month-old mature
627 Sprague Dawley sham-operated (Sham) or ovariectomized (OVX) rats were treated
628 with either vehicle, 17ß-estradiol (2.0 mg/kg.day), tamoxifen (Tamo-L, 0.1 mg/kg.day
629 and Tamo-H, 1.0 mg/kg.day), DBT (3.0 g/kg.day) and combinations of DBT and
630 tamoxifen for 12 weeks. Breast tissue was collected upon sacrifice and the morphology
631 of breast tissue (100X) was visualized by H&E staining.

633 **Figure 4 Estrogenic effects of DBT, tamoxifen and their combinations on bone**
634 **microarchitecture, bone mineral density and mRNA expression in bone tissue of**
635 **mature ovariectomized rats** Six-month-old mature Sprague Dawley sham-operated
636 (Sham) or ovariectomized (OVX) rats were treated with either vehicle, 17ß-estradiol
637 (2.0 mg/kg.day), tamoxifen (Tamo-L, 0.1 mg/kg.day and Tamo-H, 1.0 mg/kg.day),
638 DBT (3.0 g/kg.day) and combinations of DBT and tamoxifen for 12 weeks. Bone
639 microarchitecture (**A**) and bone mineral density (BMD) of lumbar vertebra (**B**) were
640 measured by Micro-CT.   Interleukin-6 (*IL-6*) (**C**) and alkaline phosphatase *(ALP)*
641 mRNA expression (**D**) in tibial head were measured were determined by real-time PCR.
642 Data was expressed as mean ± SEM. n=5 to 12. ***$p<0.001$ vs sham; ^^$p<0.01$,
643 ^^^$p<0.001$ vs OVX.

645 **Figure 5 Estrogenic effects of DBT, tamoxifen and their combinations in central**
646 **nervous system of mature ovariectomized rats** Six-month-old mature Sprague
647 Dawley sham-operated (Sham) or ovariectomized (OVX) rats were treated with either
648 vehicle, 17ß-estradiol (2.0 mg/kg.day), tamoxifen (Tamo-L, 0.1 mg/kg.day and Tamo-
649 H, 1.0 mg/kg.day), DBT (3.0 g/kg.day) and combinations of DBT and tamoxifen for 12
650 weeks. Striatum was collected upon sacrifice. mRNA expression of tyrosine
651 hydroxylase (**A**) and dopamine transporter (DAT) (**B**) were measured by real-time PCR.
652 Data was expressed as mean ± SEM. n=5 to 12. ***$p<0.001$ vs sham; ^$p<0.05$, ^^$p<0.01$,
653 ^^^$p<0.001$ vs OVX.

655 **Figure 6 Direct estrogenic effects of DBT and tamoxifen on estrogen response**
656 **element (ERE) luciferase activity in ER-positive cells** Human breast cancer MCF-7
657 (**A**), endometrial cancer Ishikawa (**B**), neuroblastoma SHSY5Y (**C**) and osteosarcoma
658 MG-63 cells (**D**) were seeded in 24-well plate and transfected with 0.4 μg of ERETkluc

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plasmid and subjected to treatment with DBT or tamoxifen for 24 hr. ERE luciferase activity were measured by Dual Luciferase® Reporter Assay System. Results were from two independent experiments and expressed as ratio to control. n=3 or more. $^*p<0.05$, $^{**}p<0.01$, $^{***}p<0.001$ vs control.

Figure 7 Direct estrogenic effects of DBT, tamoxifen and their combinations in ER-positive cells Human breast cancer MCF-7 (**A**), endometrial cancer Ishikawa (**B**), neuroblastoma SHSY5Y (**C**) and osteosarcoma MG-63 cells (**D**) were routinely cultured and treated with DBT, tamoxifen and their combinations for 48 hr. cell viability or ALP activity were measured by MTS assay or ALP assay. Results were from two independent experiments and expressed as ratio to control. n=3 or more. $^*p<0.05$, $^{**}p<0.01$, $^{***}p<0.001$ vs control; $^{\wedge}p<0.05$, $^{\wedge\wedge}p<0.01$, $^{\wedge\wedge\wedge}p<0.001$ vs tamoxifen alone.

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