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ORIGINAL ARTICLE

Network Pharmacology Analysis and Experimental Validation of the Antidepression Mechanism of Lactuside B

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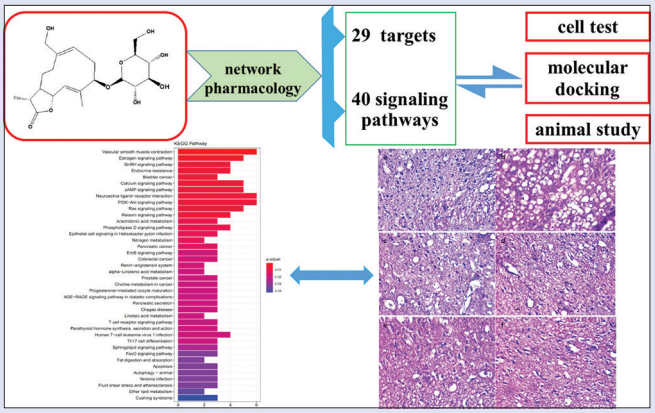
ABSTRACT

Background: Lactuside B (LB) is a novel active ingredient separated from *Pterocypsela elata* by our team. In our preliminary study, LB showed a therapeutic effect on depression. However, the underlying molecular mechanisms remain unclear. **Objectives:** This study was the first to study the pharmacological effects and mechanisms of LB on depression. **Materials and Methods:** Network pharmacology method was applied to screen the candidate targets and signaling pathways. Furthermore, molecular docking, cell test, and animal experiments were used to confirm the antidepression mechanisms. **Results:** The network pharmacology results showed 29 targets and 40 signaling pathways of LB on depression. Molecular docking showed that there was a strong binding effect between LB and each target (cAMP response element binding protein B [CREB], Ras, Raf, and ERK1/2). Cell tests indicated that the expressions of Ras and CREB in astrocyte cells (0.05–0.20 g/L of LB) exhibited significant differences ($P < 0.05$) compared with the model group, respectively. In animal tests, the expressions of Ras, ERK1/2, and Raf (12.5–50.0 μg/10 g of LB) showed a significant difference compared with mice in the model group ($P < 0.05$). **Conclusion:** These results indicated that the antidepression mechanism of LB was mainly associated with Ras signaling pathway, and the cAMP signaling pathway and PI3K-Akt signaling pathway may play an essential role.

Key words: Depression, lactuside B, molecular mechanism, network pharmacology, target

SUMMARY

- This study was the first to study the pharmacological effects and mechanisms of LB on depression. Network pharmacology method was applied to screen the candidate targets and signaling pathways. Molecular docking, cell test, and animal experiments were used to confirm the mechanisms.



Abbreviations used: BP: Biological process; CC: Cellular component; LB: Lactuside B; MF: Molecular function; CREB: cAMP response elementbinding protein B.

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INTRODUCTION

Pterocypsela elata (Hemsl.) Shih (Asteraceae) has historically been widely used as a vegetable (young stems and leaves) and herb (*Pterocypsela elata*).[1] It has multiple pharmacological properties, such as reducing fever and removing toxicity, promoting blood circulation, anti-inflammatory, antioxidant, and so on.[1,2] In our previous studies, more than 14 ingredients were extracted and separated from *Pterocypsela elata*.[2-7] Particularly, Lactuside B [LB, chemical structure shown in Figure 1a] is a novel and main active ingredient of *Pterocypsela elata*. With the good therapeutical effect of LB on cerebral ischemia and myocardial ischemia,[1-5] we had got the patent of China (No. CN20061011376.0). Our preliminary study had shown that LB had a good treatment effect on depression.

As a major public health problem associated with a high morbidity and suicide rate, depression has a strong impact on the quality of life.[8-12] Globally, it was estimated that there were more than 300 million people affected by depression. In China, it had ranked first place in the total burden of all the diseases.[12,13] Although many antidepressants had been used in clinical practice, there were some major clinical concerns, such as a significant time lag, individual difference, significant or sustained remission with a single antidepressant drug, and so on.[13] So, a novel antidepressant was urgently needed currently. With a novel chemical structure, LB may have a good prospect in the treatment of depression. However, its underlying molecular mechanism of antidepression remains unclear.

With the advantages of a holistic perspective and biological networks, network pharmacology was a good method to study the mechanisms of drugs. Network pharmacology could explore the complex interactions between drug and disease, and provide a new approach for drug screening and identifying multiscale mechanisms from a network perspective at the molecular level.[14-18] In recent years, our groups had successfully used

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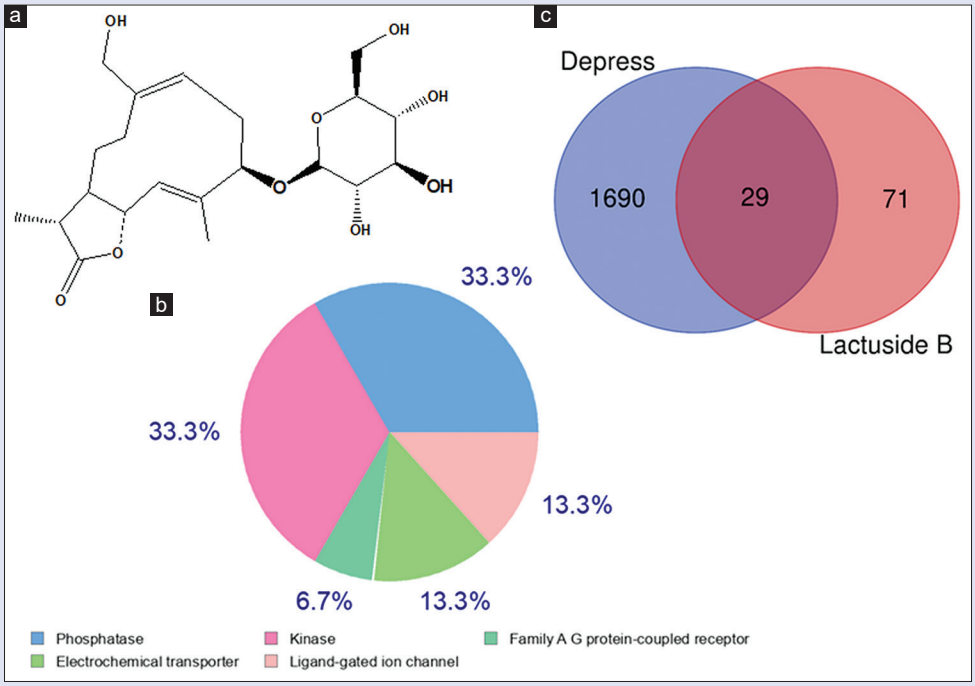

Figure 1: The structural formula of LB (a), the distribution of candidate targets for LB (b) and the intersection of targets related to depression and LB (c)

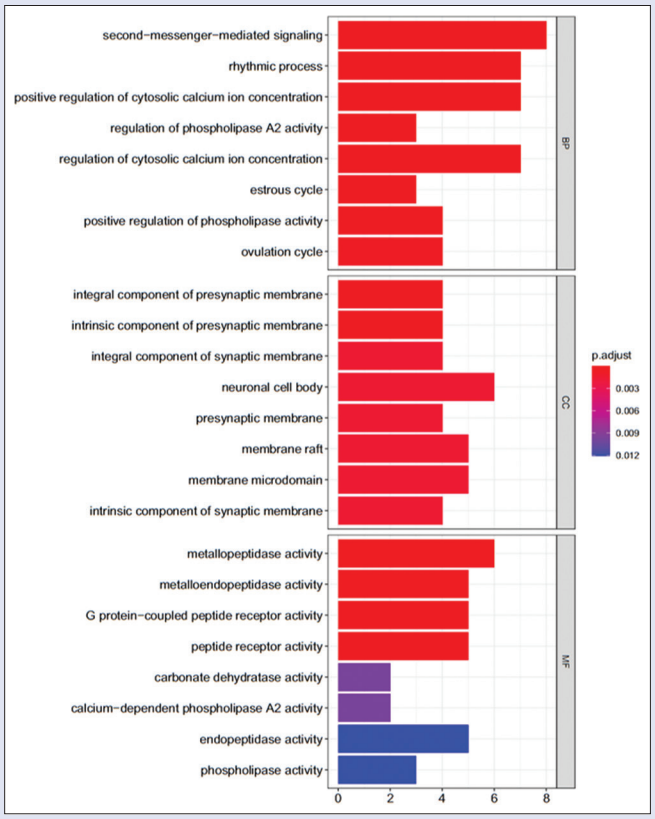

Figure 2: The gene ontology enrichment enriched analysis for biological process (BP), molecular function (MF), and cellular component (CC)

the network pharmacology method to explore bioactive ingredients, potential targets, and signaling from *Isodon rubescens*,[15] *Pterocypsela elata*,[1] and Lian-Ge granules,[16] respectively.

To our knowledge, the pharmacological and phytochemical studies on *Pterocypsela elata* have not been reported except for those from our team. In our preliminary study, LB showed a therapeutic effect on depression. However, the underlying molecular mechanisms remain unclear. In this study, the pharmacological effects and mechanisms of LB on antidepression were firstly studied. Based on the network pharmacology method, the candidate targets and signaling pathways were explored for LB. Furthermore, the cell test and mice of depression were used to evaluate and confirm the mechanisms of LB on antidepression.

MATERIALS AND METHODS

Reagents

LB (purity >99%) was extracted and separated from *Pterocypsela elata* by our research team according to the patented method (Patent of China, No. CN20061011376.0). Mouse Anti-CREB1 (No. BM17600), Anti-Ras Antibody (No. BM4940), SDS-PAGE protein-loading buffer (No. P0015), and BeyoECL Plus solution (No. P0018S) were purchased from Beyotime Biotechnology Co., Ltd. (Shanghai, China). Anti-ERK1 + ERK2 (No. ab54230) was provided by Abcam (Shanghai) Trading Co., Ltd. (Shanghai, China). Trypsin and EDTA (No. GNM25200) were gained from Ji'nuo Biomedical Co., Ltd. (Hangzhou, China).

Network pharmacology

The Swiss Target Prediction Database (http://www.swisstargetprediction.ch/index.php) was used to analyze the active targets of LB. The DrugBank database (Version 5.1.0, https://go.drugbank.com/) and DisGeNET Database (Version 6.0, https://www.disgenet.org/home/) were used to screen the therapeutic targets of antidepression. After confirming by intersection analysis, the obtained targets were regarded as the candidate therapeutic targets of LB on depression.

Then, the KEGG database (http://www.kegg.jp/) and String database (Version 11.0, http://www.string-db.org/) were used to analyze the biological process (BP), molecular function (MF), cellular

component (CC), and KEGG pathway with the above-identified targets. In this process, the smaller the value of *P*-adjust was, the greater the significant difference the corresponding biological processes would get.

Molecular docking

The docking simulation of LB and the targets were performed via the molecular docking method. The chemical structure of LB was drawn with the ChemBioOffice software (ChemBioDraw and ChemBio3D, Ultra 14.0). The protein structures of the targets were firstly obtained from the RCSB Protein Data Bank (PDB) archive (https://www.rcsb.org/). Then, for each protein, all water molecules were removed by adding the polar hydrogen and the Gasteiger charges. The geometry of DCD was subsequently optimized to minimal energy. Finally, the docking was carried out by Autodock Vina software (Version 1.1.2) and Autodock Tools (Version 1.5.6) with the default value for each parameter. For the possible conformations of LB bound with the targets, the docking conformation that had the lowest binding free energy was studied, and the interaction with active site residues was analyzed by the PyMoL software (Version 1.7.2.1).

Cell experiments

The astrocyte cells were obtained from the Cell Bank of Typical Cultures Preservation Committee of the Chinese Academy of Sciences (Shanghai, China). When the cells were in logarithmic condition, they were randomly divided into six groups: four groups were treated with 0.025 g/L, 0.50 g/L, 0.10 g/L, and 0.20 g/L of LB, respectively; the model group was treated with potassium chloride solution with no treating for the control group. After 24 h of treatment, the expressions of Ras and CREB were detected with six multiple holes for each group by Western blot analysis, respectively.

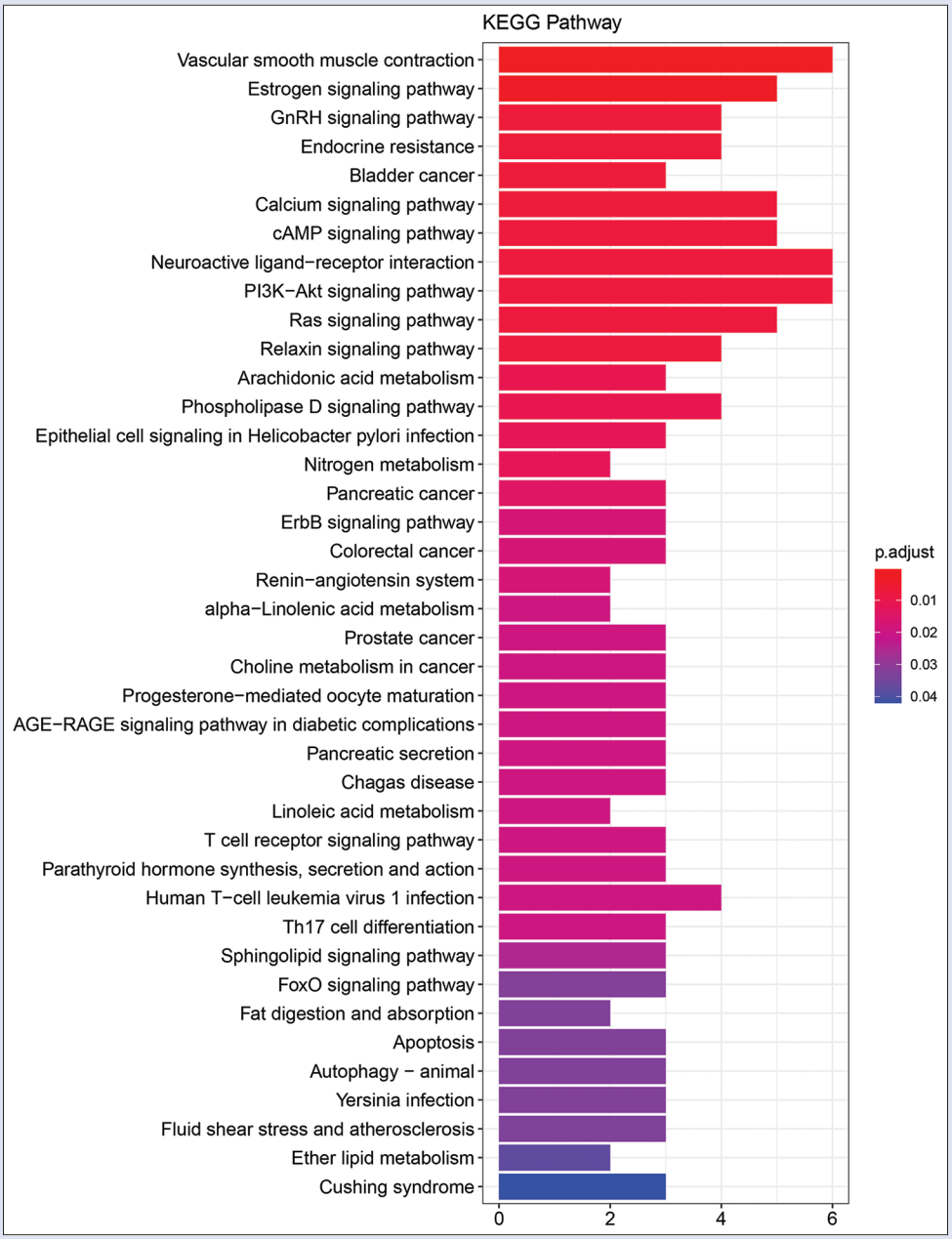

Figure 3: The potential KEGG pathways of LB on depression

Animal experiments

The male Balb/C mice (weighing 20–25 g) were purchased from the Laboratory Animal Center of Hualan Bioengineering Co., Ltd. (Xinxiang, China). These mice were adapted to a standard environment (chow and water available *ad libitum*, alternating between 12 h of daylight and 12 h of darkness) for 1 week. One week later, except for the mice in the control group, the remaining mice were induced into depression with several manipulations, such as reversing the light-dark period, cage tilting (45° along the vertical axis), noise, physical restraint, tail clamping, food depriving, and water depriving with an empty water bottle. After these manipulations were carried out in a random order for three consecutive weeks, the model mice were confirmed with the open field test, forced swimming test, and sucrose preference test, respectively.[13,19,20] Then, the depression mice were randomly divided into the following five groups. Mice in the three LB groups were administrated with 12.5 μg/10 g, 25.0 μg/10 g, and 50.0 μg/10 g of LB by gavage, respectively; the fluoxetine group was injected intraperitoneally with 10 μg/10 g of fluoxetine. The mice in the control and model group were treated with 10 μg/10 g of normal saline. After 4 week of administration, the histological changes and the expressions of Ras, ERK1/2 and Raf in cortex tissue were analyzed, respectively.

The experiment was approved by the Animal Ethics Committee of Xinxiang Medical University (No. SCXK-Yu-2010-0001). The experimental procedures were followed by the ARRIVE guidelines, the U.K. Animals (Scientific Procedures) Act, 1986 and associated guidelines, EU Directive 2010/63/EU for animal experiments, or the National Research Council's Guide for the Care and Use of Laboratory Animals.

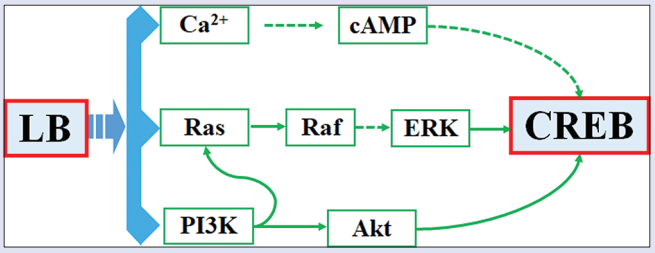

Figure 4: The 3 signaling pathways of LB on depression

Statistical analysis

In this study, all data were analyzed in the form of mean ± standard deviation by the Statistical Package for the Social Sciences (SPSS) 13.0 software (SPSS Inc., Chicago, IL, USA). In the statistical comparison, $P < 0.05$ was considered as statistical significance while $P < 0.01$ indicated extreme statistical significance.

RESULTS AND DISCUSSION

Candidate targets and signaling pathways

After searching the structure of LB [as shown in Figure 1a] in the Swiss Target Prediction Database, 71 active targets were activated by LB. As shown in Figure 1b, these candidate targets were mainly distributed in Phosphatse (33.3%) and Kinase (33.3%). In the DrugBank database and DisGeNET Database, 1690 therapeutic targets of depression were

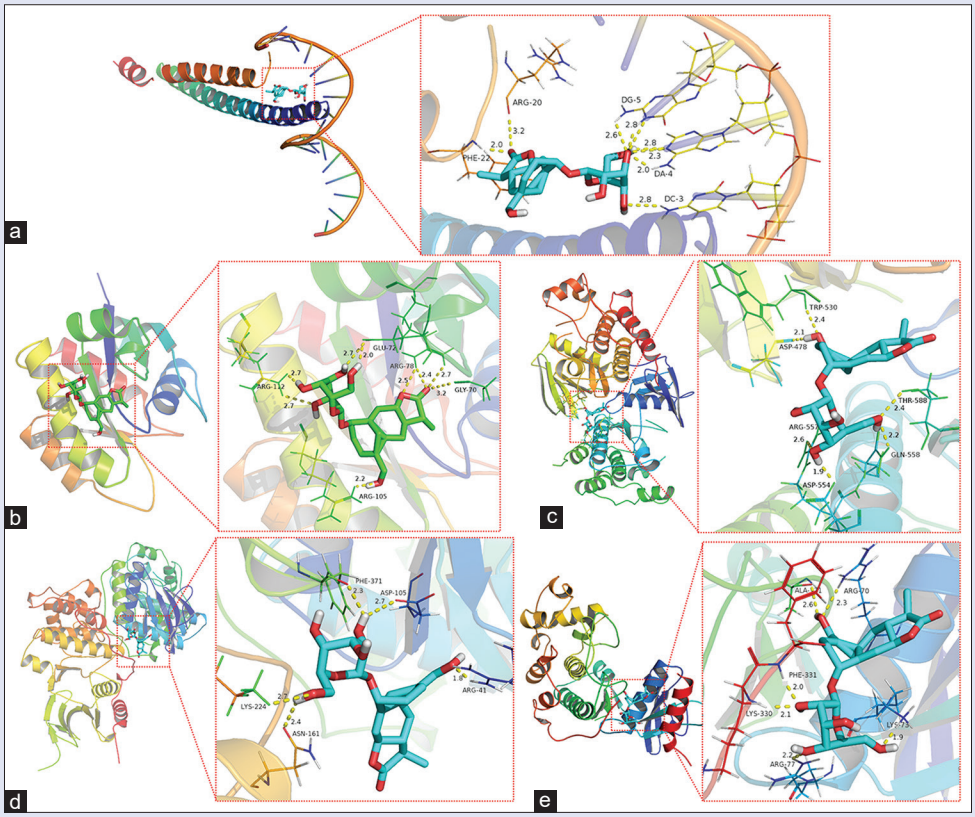

Figure 5: The docking results of LB with CREB (a), Ras (b), Raf (c), ERK1 (d), and RRK2 (e) by 3-D mode, respectively. The structures of proteins were shown in cartoon model while LB with stick model and amino acid residues with line model (the number showed the site in the protein). The hydrogen bonds between LB and each amino acid residue were represented in yellow dashed lines with the number showed the distance (Å)

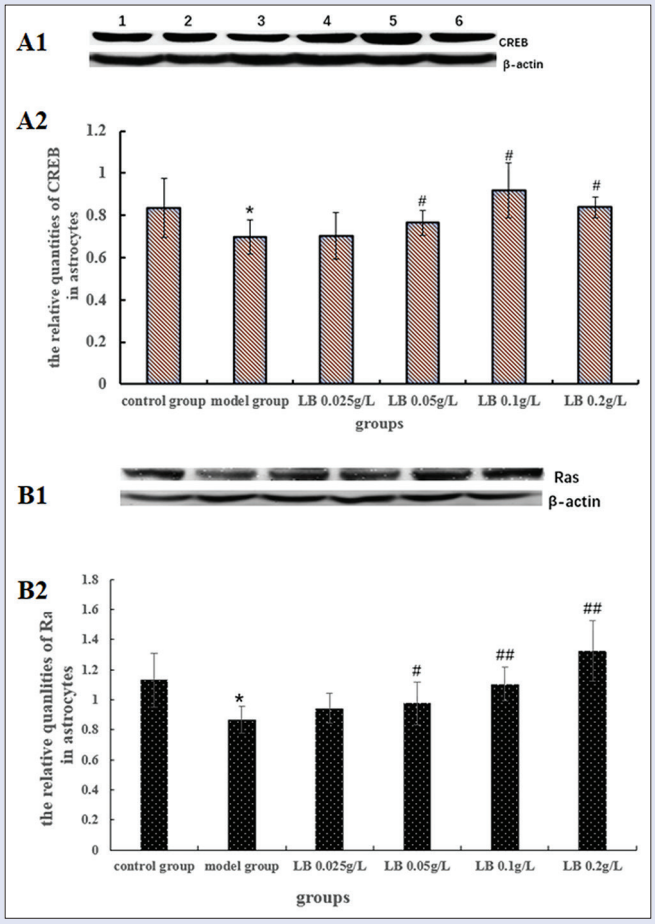

Figure 6: The effect of LB on the expressions of CREB (a) and Ras (b) in astrocytes ($n = 6$, lane1-6 of A1 and B1: the protein expression in astrocytes of the control group, model group, 0.025 g/L LB group, 0.05 g/L LB group, 0.10 g/L LB group, and 0.20 g/L LB group. A2 and B2: the semiquantitative analysis of protein expression in astrocytes. *Significant differences between the control group and the model group, $P < 0.05$; #significant differences between the control group and LB group, $P < 0.05$; ##extreme statistical significance between the control group and LB group, $P < 0.01$)

obtained. As listed in Figure 1c, 29 targets at the intersection were regarded as the candidate targets of LB on antidepression, and Table 1 provided the information on these identified targets.

The relationships among these 29 targets were studied in the gene ontology enrichment analysis by the String database, and enriched in the BP, MF, and CC, respectively. As shown in Figure 2, there were eight clusters for BP, MF, and CC (*P*-adjust <0.05), respectively. After analysis of the KEGG pathways by the KEGG database, 40 potential KEGG pathways [as listed in Figure 3] were obtained for LB in the treatment of depression (*P*-adjust <0.05).

In the cell tests or animal experiments, it may be unrealistic to confirm the above potential pathways in the way of one by one. As indicated in Figure 4, the cAMP response element-binding protein B (CREB) was a common and important downstream protein in the following three pathways including cAMP signaling pathway, PI3K-Akt signaling pathway and Ras signaling pathway.[21,22] So, these three potential KKGG pathways have attracted our interests, the docking simulation, the cell tests, and the animal experiments were performed to confirm the mechanism of LB on depression.

Table 1: The information of 29 potential targets of LB on depression

Targets	Protein names	Uniprot ID
MMP2	72 kDa type IV collagenase	P08253
MMP7	Matrilysin	P09237
PLA2G2A	Phospholipase A2, membrane associated	P14555
P2RX3	P2X purinoceptor 3	P56373
ADORA2A	Adenosine receptor A2a	P29274
EDNRA	Endothelin-1 receptor	P25101
CXCR2	C-X-C chemokine receptor type 2	P25025
OPRK1	Kappa-type opioid receptor	P41145
AGTR1	Type-1 angiotensin II receptor	P30556
PDE4D	cAMP-specific 3',5'-cyclic phosphodiesterase 4D	Q08499
MME	Neprilysin	P08473
MCL1	Induced myeloid leukemia cell differentiation protein Mcl-1	Q07820
AMPD3	AMP deaminase 3	Q01432
CA12	Carbonic anhydrase 12	O43570
MMP12	Macrophage metalloelastase	P39900
KDM5B	Lysine-specific demethylase 5B	Q9UGL1
PLA2G1B	Phospholipase A2	P04054
EGFR	Epidermal growth factor receptor	P00533
IL2	Interleukin-2	P60568
LGALS3	Galectin-3	P17931
MAPK8	Mitogen-activated protein kinase 8	P45983
BMP1	Bone morphogenetic protein 1	P13497
CA2	Carbonic anhydrase 2	P00918
HSP90AB1	Heat shock protein HSP 90-beta	P08238
PPP2CA	Serine/threonine-protein phosphatase 2A catalytic subunit alpha isoform	P67775
OPRM1	Mu-type opioid receptor	P35372
SLC5A2	Sodium/glucose cotransporter 2	P31639
MAP2K1	Dual specificity mitogen-activated protein kinase kinase 1	Q02750
LTA4H	Leukotriene A-4 hydrolase	P09960

Table 2: The docking information of LB and the four targets

Targets	PDB ID	Affinity (kcal/mol)	Amino acid residue (site in protein)
CERB	5ZK1	−7.5	DC (3), DA (4), DG (5), PHE (22), ARG (20)
Ras	1X1R	−7.0	GLY (70), GLU (72), ARG (78, 112, 105)
Raf	1UWJ	−7.7	ASP (478, 554), TRP (530), ARG (557), GLN (558), THR (588)
ERK1	2ZOQ	−7.9	ARG (41), ASP (105), ASN (161), LYS (224), PHE (371)
ERK2	6RQ4	−7.4	ARG (70, 77), ALA (71), LYS (73), LYS (330), PHE (331)

Molecular modeling study

For each target, the protein structure was obtained from the PDB database. Table 2 listed the affinity and binding amino acid residues between LB and each target (CREB, Ras, Raf, ERK1, and RRK2), and the interaction modes were shown in Figure 5. The value of affinity indicated the calculated binding energy between the active ingredient and the protein. As the affinity is less than −7.0 kcal/mol, it means there is strong binding energy. So, LB had strong binding interaction with each target as listed in Table 2. As displayed in Figure 5, the LB molecule was located in the cavity of each target. The hydrogen bonds indicated the LB molecule was surrounded by the amino acid residues, which indicated the

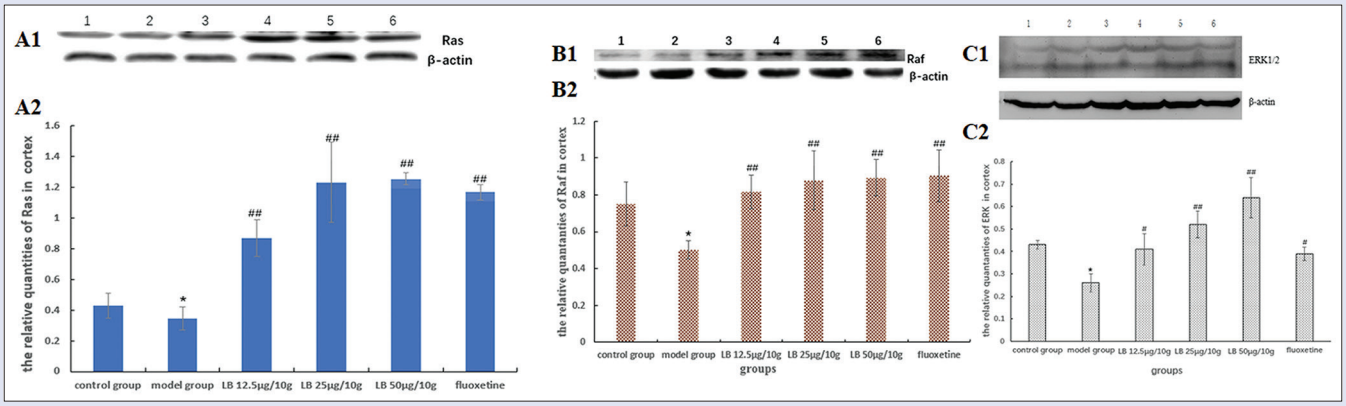

Figure 7: The effect of LB on the expressions of Ras (a), Raf (b), and ERK1/2 (c) in cortex tissues (n = 3, lane1-6 of A1, B1, and C1: the protein expression in the control group, model group, 12.5 μg/10 g of LB group, 25.0 μg/10g of LB group, 50.0 μg/10 g of LB group, and fluoxetine group. A2, B2, and C2: the semiquantitative analysis of protein expression. *Significant differences between the control group and the model group, $P < 0.05$; #significant differences between the model group and LB group or fluoxetine group, $P < 0.05$; ##extreme statistical significance between the model group and LB group or fluoxetine group, $P < 0.01$)

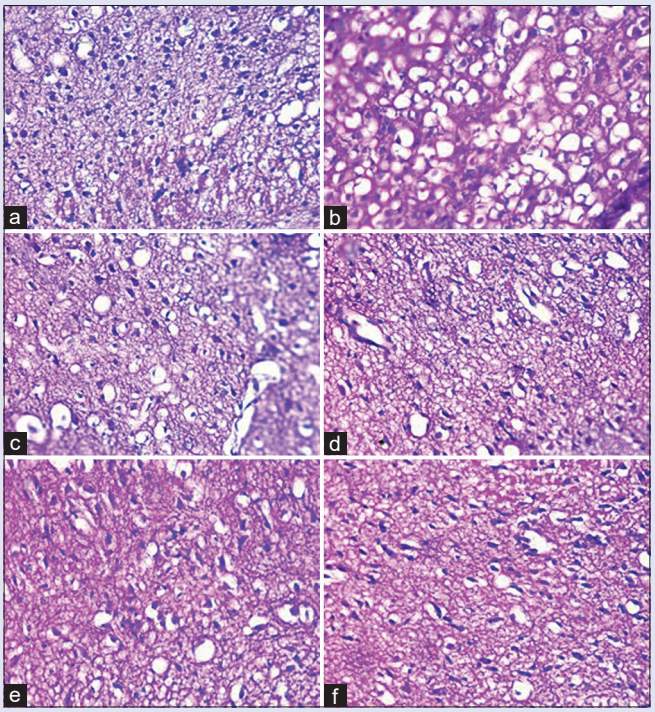

Figure 8: The effect of different dosages of LB on morphological changes of cortex tissues (×100) (a: control group; b: model group; c: LB 12.5 μg/10 g group; d: LB 25 μg/10 g; e: LB 50 μg/10 g group; f: fluoxetine group)

optimal binding site of each target for the LB molecular, so it could play an essential effect in increasing the binding affinity of LB to each target.

Cell test

CREB is a general transcription activator, which is widely expressed in all nucleated cells. As shown in Figure 6a, compared with the control group, the expression of CREB in the model group decreased significantly ($P < 0.05$). However, cells treated with LB at the concentration of 0.05–0.20 g/L could improve the expression of CREB significantly compared with that in the model group ($P < 0.05$). Furthermore, 0.10 g/L of LB could increase the expression of CREB to a similar or higher level in the control group. So, the three signaling pathways (cAMP signaling pathway, PI3K-Akt signaling pathway, and Ras signaling pathway) might be the mechanisms of LB on antidepression.

As listed in Figure 4, the Ras protein was related with the PI3K-Akt signaling pathway and Ras signaling pathway.[23-27] The expression of Ras was analyzed to further explore the mechanisms. As revealed in Figure 6b, there was a dose-dependent relationship between the expression of Ras and the concentration of LB at 0.025–0.20 g/L. Importantly, 0.05 g/L of LB could significantly increase the expression of Ras ($P < 0.05$) while 0.10 g/L and 0.20 g/L of LB could improve that very significantly ($P < 0.01$). So, these results were partly consistent with that of the network pharmacology, and greatly enhanced the understanding of the mechanisms of LB on antidepression.

Animal study

In the animal experiments, a serial of proteins (Ras, Raf, and ERK) were studied comprehensively for the RAS signaling pathway as shown in Figure 4. As indicated in Figure 7, the expressions of Ras, ERK1/2, and Raf in mice cortex tissues of the model group were decreased significantly compared with those in the control group ($P < 0.05$), respectively. As revealed in Figure 7a and 7b, after 4 week of administration with different concentrations of LB, the expressions of Ras and Raf in mice cortex tissues had been increased very significantly compared with those of the model group ($P < 0.01$), respectively. Furthermore, 25.0 μg/10 g–50.0 μg/10 g of LB could improve the expressions of Ras and Raf to a similar level in the fluoxetine group. For the expression of ERK1/2 [Figure 7c], significant differences also were observed between the different concentrations of LB groups and the model group ($P < 0.05$). So, LB could upregulate the RAS signaling pathway with have a good effect on antidepression.

The tissue slices of the mice cortex were prepared with an H and E tanning method [as shown in Figure 8]. As seen in Figure 8a, the cortical cells of the control group were arranged orderly and evenly, and these cells were of uniform sizes and regular shape. On the other hand, the cells of the model group [Figure 8b] showed big vacuoles and swelling, and the cells were arranged unevenly with indistinct boundaries. After the administration of LB or fluoxetine, the vacuoles

and swelling of these groups [Figure 8b -8f] was weakened. Especially in the 50 μg/10 g of LB group and fluoxetine group, the number of swelling cells was significantly reduced.

Based on the results of the cell test and animal experiment, it could be confirmed that the RAS signaling pathway was an important mechanism for LB in antidepression. The cAMP signaling pathway and the PI3K-Akt signaling pathway may also play an essential role. Overall, this study solidly supported the therapeutic effects of LB on antidepression.

CONCLUSION

As a novel active ingredient of *Pterocypsela elata*, LB had a good therapeutic effect on depression. Network pharmacology showed that it had 29 targets and 40 signaling pathways for antidepression. The cell tests indicated that the Ras and CREB expression exhibited significant difference ($P < 0.05$) in astrocyte cells treated with LB (0.05–0.20g/L) compared with the model group, respectively. In animal tests, the Ras, ERK1/2, and Raf expression showed significant differences in mice treated with LB (12.5–50.0 μg/10 g) compared with mice in the model group ($P < 0.05$). The cell and animal studies indicated that the antidepression mechanism of LB was mainly associated with Ras signaling pathway, and the cAMP signaling pathway and PI3K-Akt signaling pathway may play an essential role. We would carry out further exploration in the following study. Overall, this study solidly supported the therapeutic effects of LB on antidepression. Meanwhile, this approach provided a powerful means to explore the mechanism of LB and other herbs.

Acknowledgements

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Conflicts of interest

There are no conflicts of interest.

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