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Eupolyphaga sinensis Walker displays inhibition on hepatocellular carcinoma through regulating cell growth and metastasis signaling

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Tumor growth and metastasis are responsible for most cancer patients' deaths. Here, we report that *eupolyphaga sinensis* walker has an essential role in resisting hepatocellular carcinoma growth and metastasis. Compared with proliferation, colony formation, transwell assay and transplantable tumor in nude mouse in vitro and vivo, *eupolyphaga sinensis* walker extract (*ESWE*) showed good inhibition on the SMMC-7721 cell growth and metastasis. Using genome-wide microarray analysis, we found the down-regulated growth and metastasis factors, and selected down-regulated genes were confirmed by real-time PCR. Knockdown of a checkpoint PKC β by siRNA significantly attenuated tumor inhibition and metastasis effects of *ESWE*. Moreover, our results indicate *ESWE* inhibits HCC growth by not only downregulating the signaling of PKC β , Akt, m-TOR, Erk1/2, MEK-2, Raf and JNK-1, but also increasing cyclin D1 protein levels and decreasing amount of cyclin E, cyclin B1 and cdc2 of the cycle proteins. At the same time, *ESWE* reduced MMP2, MMP9 and CXCR4, PLG, NF κ B and P53 activities. Overall, our studies demonstrate that *ESWE* is a key factor in growth and metastasis signaling inhibitor targeting the PKC, AKT, MAPK signaling and related metastasis signaling, having potential in cancer therapy.

Tumor cell proliferation is closely related to the cell cycle and tumor cell metastasis, and therefore induction of cell cycle arrest and inhibition on metastasis are an effective method of controlling tumor cell growth¹. Tumor metastasis is a complex process involving several key steps, during the process, tumor cell migration and invasion are the two critical steps and responsible for the entry of tumor cells into blood vessels and lymph nodes². These include cell adhesion, invasion, proliferation and vessel formation³. There are various molecular players and signaling cascades involved in the proliferation and metastasis pathway, such as the phosphatidylinositol 3-kinase (PI3K)/AKT, Ras/Raf/mitogen-activated protein kinase (MAPK), phospholipase-C γ /protein kinase C (PLC γ /PKC) pathway, cyclins, cyclin-dependent kinases (CDKs), matrix metalloproteinases (MMPs) and endogenous CXC chemokine receptor-4(CXCR4), etc. These signaling pathways regulate important cellular functions including cellular proliferation, migration, cell cycle and apoptosis^{4,5}.

Hepatocellular carcinoma (HCC) is the fifth most common solid tumor in the world and the third most common cause of cancer mortality⁶. Despite significant advances in early detection and therapy, HCC is still one of the leading causes for cancer-related death worldwide⁷. Tumor recurrence in HCC can occur as metastases, whereas more than 90% of HCC-related deaths are the result of secondary local or distant diseases. Systemic pharmacotherapy is the main treatment for those patients. Recently, drugs targeting key pathways have generated new perspectives in the field of the treatment of HCC⁶.

However, efficacious or curative drug therapy for HCC and its metastases remains elusive⁷. There is an urgent need for more effective agents for the clinical management of HCC. Many traditional Chinese herbs are promising drugs for cancer therapy because of both their potential as chemopreventive agents and their chemotherapeutic activities against HCC in experimental studies⁸. *Eupolyphaga sinensis* Walker (*ESW*) is one of numerous insects commonly used as a food and has been used in Chinese traditional medicine for a long time⁹. As a spice in southeast Asia, including China, Thailand, India and Malaysia¹⁰, it has been used to treat many different diseases such as ecchymoma, post-traumatic wounds, hepatic fibrosis and tumors in clinical practice¹¹. Previous studies have demonstrated that *ESW* has potential in curing leukemia by removing blood stasis and promoting blood



circulation from the perspective of Chinese medicine¹². But, there have been few reports about its inhibition on HCC. The present study aimed to extend the previous study of ESW and to evaluate its inhibition on HCC growth through arrest cell cycle and metastasis inhibition using proliferation, colony formation, transwell assay, siRNA assay and transplantable tumor in nude mouse *in vitro* and *in vivo*. All the results suggest that ESW could inhibit hepatocellular carcinoma activity by regulating PKC, AKT, MAPK signaling and related metastasis signaling, and be a useful therapeutic candidate for HCC intervention.

Results

ESWE suppressed liver cancer cell proliferation and colony formation. To evaluate the effect of ESWE on liver cancer cells, we observed its action on cell proliferation and colony formation. The results showed that ESWE significantly inhibited cell proliferation in SMMC-7721, BEL-7402 and Hep G2 cells. The IC₅₀ was 0.13 mg/mL, 0.14 mg/mL and 0.67 mg/mL, respectively, and the IC₅₀ of L-02 cell was 45.42 mg/mL (Figure 1A). In colony formation assay, upon 10 ~ 15 days continuous culture, ESWE suppressed colony formation of SMMC-7721, BEL-7402 and Hep G2 cells, and ESWE showed good inhibition on the colony formation of SMMC-7721 cells related to cells of BEL-7402 and Hep G2 cells (Figure 1B–E). These findings indicate that ESWE has potential anti-tumor properties in hepatocarcinogenesis but no obvious inhibition on normal cells.

ESWE inhibits tumor growth *in vivo*. To further assess the effect of ESWE on tumor growth *in vivo*, human tumor models xenografted in athymic mice were used (Figure 2A). The anticancer effects of ESWE against SMMC-7721 transplantation tumor are shown in Figure 2B–D. Compared with the untreated group, the results showed that

treatment with ESWE resulted in significant reduction of tumor weight (the mean tumor weight were 0.54 g in the untreated group and 0.2475 g in the treated group, respectively), inhibiting tumor growth at a rate of 54.16% at 400.0 mg/kg. Furthermore, there was no change in athymic mice body weight during the experiment. The H/E staining (Figure 2E–H) showed that the tumor cells in the tissue of the control group grew fast and caused necrosis, and more mitosis in the control group indicated cell proliferation was faster than that in the ESWE treated group. So, the HE staining proves that ESWE could inhibit cell proliferation.

Differentially expressed genes in control and ESWE-treated cells.

To gain insights into the mechanisms of ESWE on inhibition to HCC, SMMC-7721 cells were cultivated in the absence or presence of 0.1 mg/mL ESWE for 48 h, followed by the Agilent Whole Human Genome Oligo Microarray. A group of differentially expressed genes obtained from the primary analysis were analyzed by GO enrichment and decrease pathway analysis, respectively. Among the down-regulated genes in ESWE-treated cells, regulation of multicellular organismal process, defense response, extracellular structure organization, protein amino acid dephosphorylation, transmembrane receptor protein serine/threonine kinase signaling pathway, salivary gland morphogenesis, lymphocyte chemotaxis, extracellular matrix organization, growth, regulation of cell proliferation, positive regulation of cell differentiation, negative regulation of immune system process, dephosphorylation, response to peptide hormone stimulus, positive regulation of developmental process, regulation of response to external stimulus, positive regulation of cell adhesion are shown in Figure 3A. The GO enrichment analysis revealed that the main GO categories for the up-regulated genes in ESWE-treated cells included response to organic cyclic substance, cell-cell signaling, vasoconstriction, response to alkaloid, response

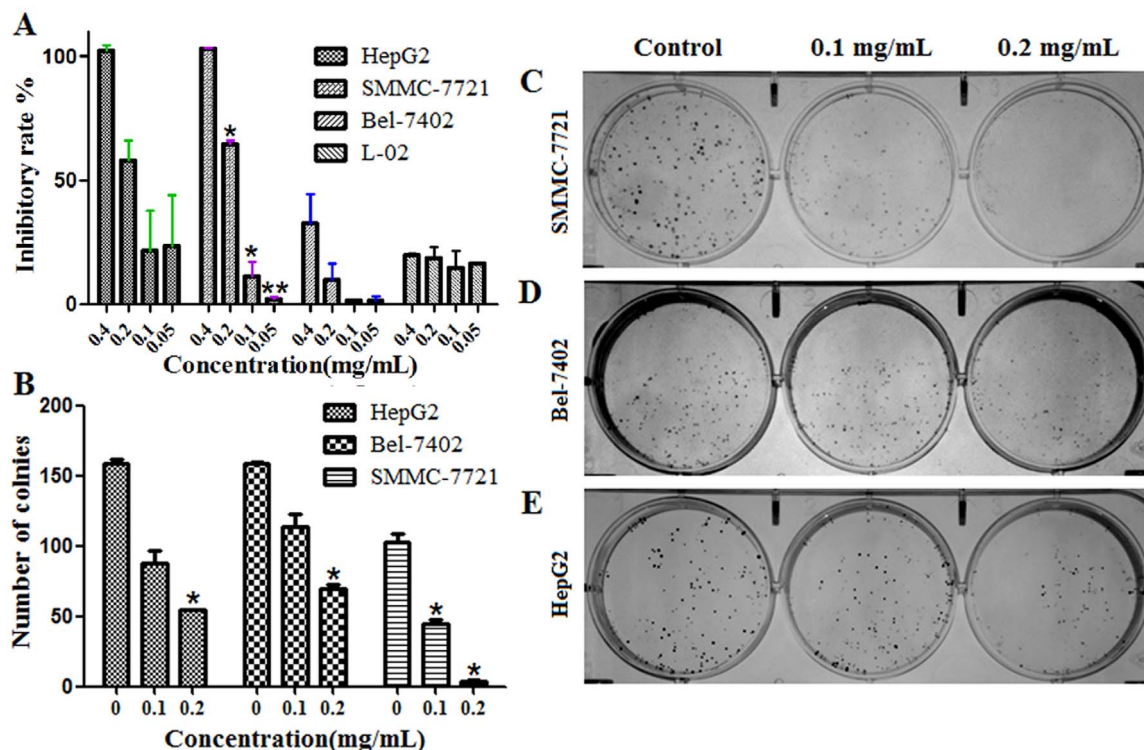


Figure 1 | ESWE suppressed liver cancer cell proliferation and colony formation. (A) Cells were treated by ESWE for 48 h at 0.05, 0.10, 0.20 mg/mL. ESWE inhibited SMMC-7721, BEL-7402 and Hep G2 cells growth *in vitro*, and had no obvious inhibition on L-02 cell. (B–E) Effect on colony formation of SMMC-7721, BEL-7402 and Hep G2 by ESWE. (B) Quantitation data of (C–E). (C–E) The representative colony formation picture of SMMC-7721, BEL-7402 and Hep G2 cell. ESWE showed better inhibition on the colony formation of SMMC-7721 than BEL-7402 and Hep G2 cells. Data were expressed as means \pm SD ($n = 5$). * $p < 0.05$, ** $p < 0.01$ vs. the untreated control group.

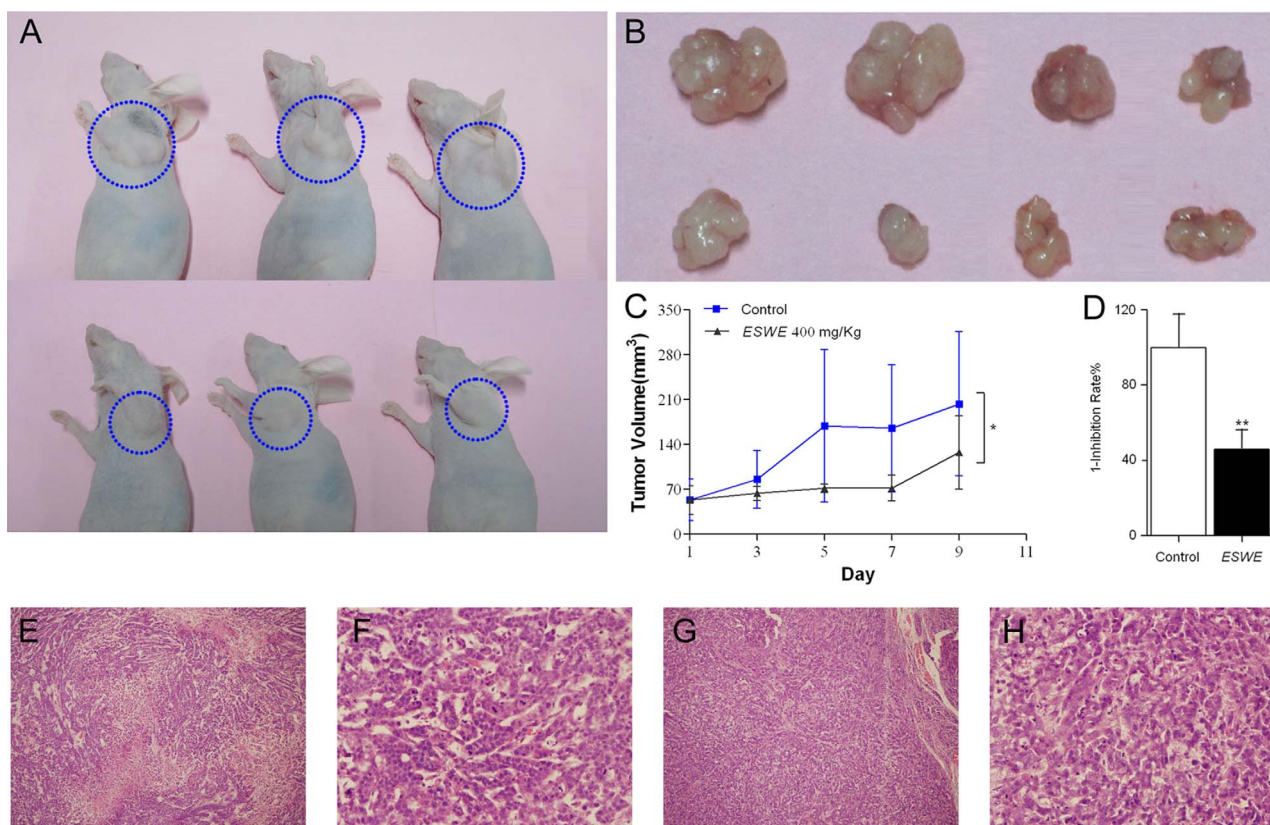


Figure 2 | Regression of live cancer xenograft by ESWE. SMMC-7721 cells were subcutaneously injected on the right hinge region of athymic nude mice. Mice were regularly observed for appearance of tumor. (A) The representative xenografts of SMMC-7721 human liver cancer in control and ESWE-treated mice. (B) The transplantable tumor, upper row is control group tumor and lower row is ESWE-treated group tumor. (C) Effect of ESWE on the tumor volume growth curve. (D) ESWE inhibited tumor growth by tumor weight. The caused necrosis in the control group (E) and ESWE-treated group (F) mice tissue by the HE staining. Effect of ESWE on the mitosis in the control group (G) and ESWE-treated group (H) mice tissue by the HE staining (400 \times magnification). Data were expressed as means \pm SD (n = 3). * p < 0.05, ** p < 0.01 vs. the control group.

to lipopolysaccharide, response to molecule of bacterial origin, chemical homeostasis, regulation of membrane potential, response to abiotic stimulus, vascular smooth muscle contraction, response to extracellular stimulus, gonad development and so on, as summarized in Figure 3B.

A heat map with two-dimensional hierarchical clustering revealed 32 genes differentially expressed between the two groups, which were down-regulated and related to growth, proliferation and metastasis, as illustrated in Figure 3C. The total genes were found to be differentially expressed between control and ESWE-treated cells at p < 0.05, and fold-change \geq 2. In this experiment, the genes responsible for growth, proliferation and metastasis were most important to match our research goal. The analysis of our microarray data showed that PLG, PKC β and IL3RA genes, associated with growth, proliferation and metastasis, were down-regulated in ESWE-treated cells as compared with the control cells. The PLG, PKC β and IL3RA genes confirmation of microarray analysis was determined by real-time PCR (Figure 3D).

ESWE arrested cell cycle and regulated related cycle proteins. The effect of ESWE on cell cycle profile was analyzed using PI staining and flow cytometry analysis. Cells were treated without ESWE or with ESWE at 0.05, 0.1, 0.2 mg/mL for 48 h and stained with propidium iodide. As shown in Figure 4A–D, treatment of ESWE induced an accumulation of SMMC-7721 cells in the G1 phase of the cell cycle. The population of untreated cells in phase G1 was 55.8%, whereas the population of cells in phase G1 treated with ESWE at 0.05, 0.1, 0.2 mg/mL was 69.71%, 76.28% and 88.13% respectively,

showing a dose-dependent increase. A decrease in the population in the G2-GM and S phases was also observed.

To elucidate the specific cell cycle regulatory proteins responsible for the cell cycle arrest, we explored the effect of ESWE on regulatory protein molecules including cyclin D1, cyclin E, and cyclin B1, which are cyclins required for advance from G1 to S, S to G2, G2 to G1 respectively. Meanwhile, we investigated the effect of ESWE on cyclin-dependent kinases cdc2. As shown in Figure 4E–F, the levels of cyclin D1 protein gradually increased with ESWE at the concentrations of 0.05–0.2 mg/mL. In contrast, the amount of cyclin E, cyclin B1 and cdc2 was significantly down-regulated in ESWE-treated cells. These data suggest that these cell cycle regulatory molecules are involved in ESWE-induced changes in cell cycle progression.

Effect of ESWE on cell growth signaling pathway. In order to clarify the inhibition and mechanism on the HCC growth by ESWE, we investigated the effect on PKC β , Akt, P-Akt, m-TOR, P- m-TOR, Erk1/2, MEK-2, Raf and JNK-1 expression of growth signaling pathway, and carried out the siRNA assay on the PKC β gene in SMMC-7721 cells (Figure 5A–C). We also compared the proliferation between normal cells and knockdown of PKC β in SMMC-7721 cells, and found that there was an obvious difference in the two groups, knockdown of PKC β significantly reduced the inhibition of ESWE on the growth of SMMC-7721 cells, as compared with the control groups transfected by a negative control shRNA (shRNA-NC) and control (P < 0.05, Figure 5D). It indicates PKC β is a key target and could affect cell growth by ESWE. Besides, ESWE

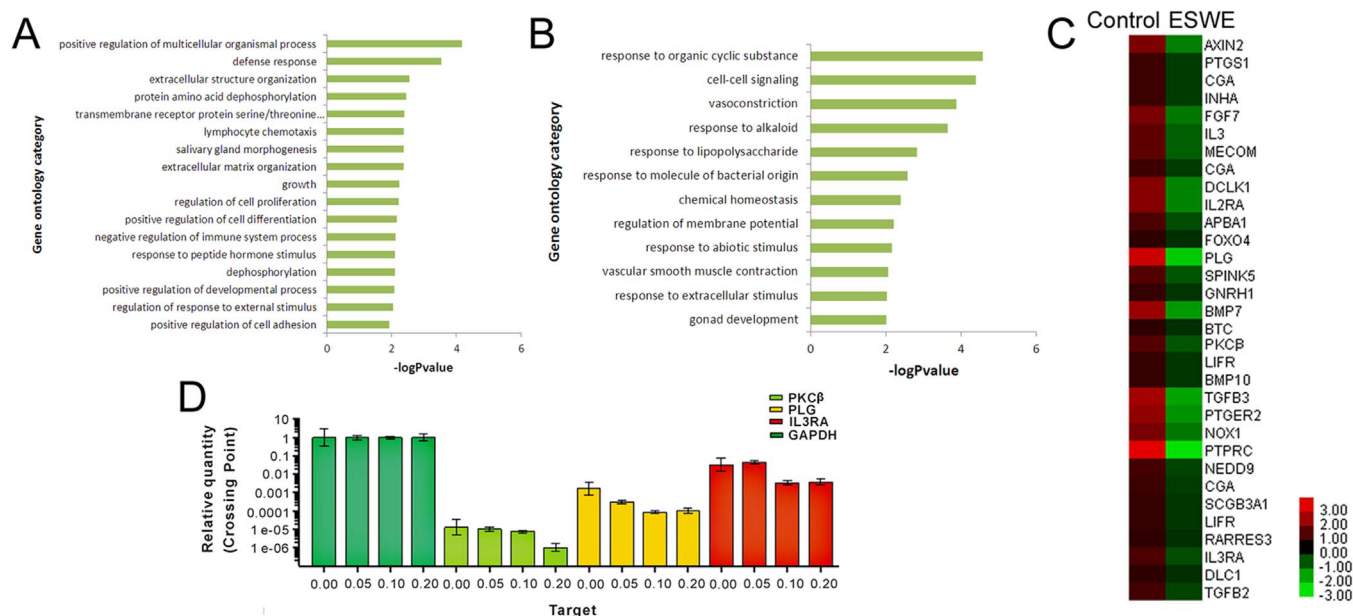


Figure 3 | The genetically different populations by ESWE for SMMC-7721 cells. (A) The GO category for the down-regulated genes in ESWE group compared with control group. (B) The GO category for the up-regulated genes in the ESWE group compared with the control group. $-\log P$ value ≥ 2 was used as a cut-off threshold to select significant GO categories. The higher the enrichment, the more significant the biological processes. (C) Hierarchical cluster analysis of 32 differentially down-regulated expressed genes for cell growth and metastasis in samples. Each column represents one sample, and each gene was depicted by one row, where red denotes an increase in gene expression and green denotes a decrease in gene expression as compared with the other group. The brighter the color, the higher the gene expression level. (D) Comparison of PKC β , PLG and IL3RA gene expression levels between control and ESWE group by real-time PCR, which confirmed the microarray analysis. Data were expressed as means \pm SD ($n = 3$). * $p < 0.05$, ** $p < 0.01$ vs. the control group.

inhibited the expressions of PKC β , Akt, P-Akt, m-TOR and P-m-TOR in the PI3K/AKT pathway (Figure 5E and F) and Erk1/2, MEK-2, Raf and JNK-1 in the Ras/Raf/MAPK pathway (Figure 5G and H) in a dose-dependent manner.

Effect of ESWE on cell migration signaling pathway. To evaluate the effect on cell migration between normal SMMC-7721 cells and PKC β knockdown cells, we observed the inhibition on cell mobility by transwell system. The results showed that treatment of ESWE significantly decreased the migration ability at the concentrations of 0.05–0.20 mg/mL, respectively, and the knockdown of PKC β significantly reduced the inhibition on the migration of SMMC-7721 cells, as compared with the control groups ($P < 0.05$, Figure 6A and B). It indicates PKC β is also a key factor in the cell migration by ESWE. Figure 6C–D showed that MMP2, MMP9 and CXCR4 protein expressions were strongly decreased in SMMC-7721 cells compared to that of control cells in a dose-dependent manner. Furthermore, the related protein PLG, NF κ B and P53 of growth and metastasis were down-regulated by treatment of ESWE ($P < 0.05$, Figure 6E and F).

Discussion

Although the extract *eupolyphaga sinensis walker* has been used for treating various human diseases, few are related to the effect on HCC, and its anti-tumor mechanism is largely unknown. The current study focuses on the evaluation of its inhibition on HCC growth through arrest cell cycle and metastasis inhibition. In this study, we showed that ESWE had a good inhibitory effect on liver cancer growth *in vivo* and *in vitro*. The molecular mechanism, in which ESWE affects cell cycle and metastasis, involves the regulation of cycle proteins, proliferation and cell migration signaling pathway.

Through *in vivo* and *in vitro* assays (Figure 1 and Figure 2), ESWE displayed good inhibition on cell proliferation, colony formation and transplantation tumor growth. To gain insights into the mechanisms

of ESWE on inhibition to HCC, microarray analysis of Agilent microarray-based gene expression profiling was used to screen the potential targets and signaling pathways (Figure 3). The major findings in microarray analysis were that the differentially expressed genes associated with growth and migration were obtained, such as PLG, PKC β and IL3RA, the selected down-regulated genes were confirmed by real-time PCR. The genes responsible for growth, proliferation and metastasis were most important to match our research goal. So we carried out potential signaling pathways analysis by ESWE.

Cancer cell growth is complicated progression which is regulated and controlled by multiple factors including cell cycle and metastasis. Cell cycle progression is tightly related to various cyclins and cyclin-dependent kinases (CDKs), such as cyclin B1, cyclin D1, cyclin E and cdk2, as well as cell proliferation¹³. Induction of cell cycle arrest is an effective method of controlling tumor cell proliferation. Cyclins are positive regulators of cell cycle progression, and cyclin gene expression is regulated in a phase-specific manner¹, such as cyclin D1 in phase G₀-G₁ and cyclin E in phase S. Both Cyclin D1 and Cyclin E are activators of the G₁/S transition¹⁴. Cyclin B1 accumulates in the S phase, reaches a maximum at mitosis, and is then rapidly degraded at the metaphase/anaphase transition. It has been established that cdc2, when associated with cyclin B, is responsible for M-phase-inductive activities^{15,16}. Flow cytometry revealed treatment of ESWE induced an accumulation of SMMC-7721 cells in the G₁ phase of the cell cycle and a decrease in the population of cells in the G₂-GM and S phase. WB assay showed the levels of cyclin D1 protein gradually increased with ESWE at the concentrations of 0.05–0.2 mg/mL. In contrast, the amount of cyclin E, cyclin B1 and cdc2 was significantly down-regulated in ESWE-treated cells. These data suggest that these cell cycle regulatory molecules are involved in ESWE-induced changes in cell cycle progression. It indicates ESWE could inhibit cell growth by arresting cell cycle caused by regulating cyclin D1, cyclin E, cyclin B1 and cdc2 expressions.

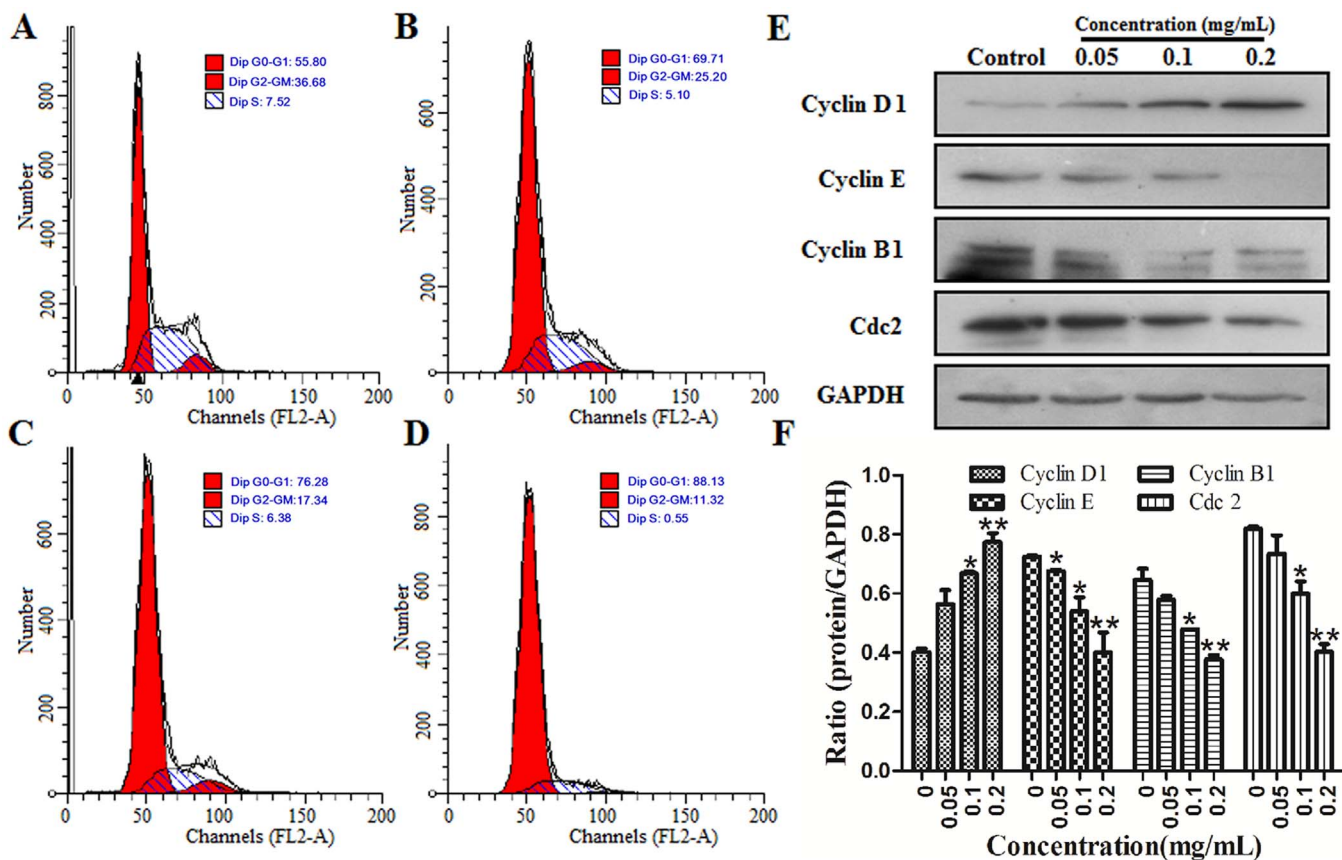


Figure 4 | *ESWE*-arrested SMMC-7721 cell cycle at the G_1 phase. Cells were treated with *ESWE* for 48 h and stained with PI. The cells were then analyzed by FACS. (A) Control. (B) 0.05 mg/mL. (C) 0.1 mg/mL. (D) 0.2 mg/mL. Data were expressed as percentage of cell population in G_0 - G_1 , S and G_2 -M phases of the cell cycle ($n = 3$). (E) Effect of *ESWE* on cycle proteins expression of Cyclin D1, Cyclin E, Cyclin B and Cdc2 by western blot analysis. Full-length blots are presented in Supplementary Figure 4E. (F) Quantitation data of (E). *ESWE* decreased the protein expressions of Cyclin E, Cyclin B and Cdc2 expressions, and increased Cyclin D1 expression in a dose-dependent manner compared to the untreated control. Data were expressed as means \pm SD ($n = 3$). * $p < 0.05$, ** $p < 0.01$ vs. the control group.

There are various molecular players and signaling cascades involved in the cellular growth pathway, such as the PKC β , Akt and m-TOR of the PLC γ /PKC, PI3K/AKT signaling pathway and the Erk1/2, MEK-2, Raf and JNK-1 of MAPK signaling pathway^{17–20}. Aberrant proliferation is likely driven by up-regulated expressions of PLC γ /PKC, PI3K/AKT and MAPK signaling pathway. Protein kinase C (PKC) is a family of serine- and threonine-specific protein kinases that can be activated by calcium and the second messenger diacylglycerol. PKC family members phosphorylate a wide variety of protein targets and are known to be involved in diverse cellular signaling pathways^{21,22}. The Akt/PKB kinase has been implicated in the genesis and/or progression of numerous human tumors, because AKT has a wide range of downstream targets that regulate endothelial cell functions such as migration, growth, proliferation, and apoptosis¹⁷. Mammalian target of rapamycin (mTOR) is a key kinase acting downstream of the activation of PI3K, and mTOR that acts as a master switch of cellular catabolism and anabolism determining tumor cell growth and proliferation¹⁹. At the same time, Erk1/2, MEK-2, Raf and JNK-1 are key checkpoints in MAPK signaling pathway. The major MAPKs including ERK and JNK are regulated by distinct signal transduction pathways that control many aspects of mammalian cellular physiology, including cell growth, differentiation and apoptosis²³. In addition, in the MAPK/ERK pathway, Raf1 becomes activated when it binds to Ras, and several MAPK kinases have been suggested to be important for phosphorylation of Raf1 as well as positive feedback phosphorylation by MAPK. This represents a conformation in which Raf1 can phosphorylate

the downstream target MEK, and this allows Raf1 to function as part of a kinase cascade, which can have profound effects on cellular proliferation and tumorigenesis^{24,25}. Our results showed *ESWE* exhibited broad spectrum inhibitory activity on the expressions of PKC β , Akt, m-TOR, Erk1/2, MEK-2, Raf and JNK-1, and targeted the PLC γ /PKC, PI3K/AKT and MAPK signaling pathway involved in tumor progression. At the same time, P-Akt and P-m-TOR were also downregulated. It provides an anti-proliferative advantage for *ESWE* to HCC associated with these signaling pathways for survival. In addition, knockdown of a checkpoint PKC β by siRNA significantly attenuated tumor inhibitory effects of *ESWE* (Figure 5), which confirmed the role of PKC β , which contributes to cell proliferation in the HCC growth, and verified the above correlated analysis.

Migration is responsible for most cancer deaths, and tumor metastasis consists of a series of sequential steps, all of which must be successfully completed. These include cell adhesion, invasion, proliferation and vessel formation^{8,26}. Hence, interruption of one or more of these steps is one approach for anti-metastatic therapy. In this study, we have found *ESWE* displayed a relation with inhibition metastasis by the down-regulated metastasis genes. Although several enzymes involved degradation of basement membrane, MMP-2 (gelatinase A) and MMP-9 (gelatinase B) play a key role in cancer cell invasion and metastasis. Therefore, inhibition of the function of MMPs, especially MMP-2 and MMP-9, is being most actively pursued for anticancer therapy^{27,28}. And also, CXCR4 plays critically important roles in cell metastasis in that decreased CXCR4 expression results in significant inhibition of cancer cell migration²⁹. We

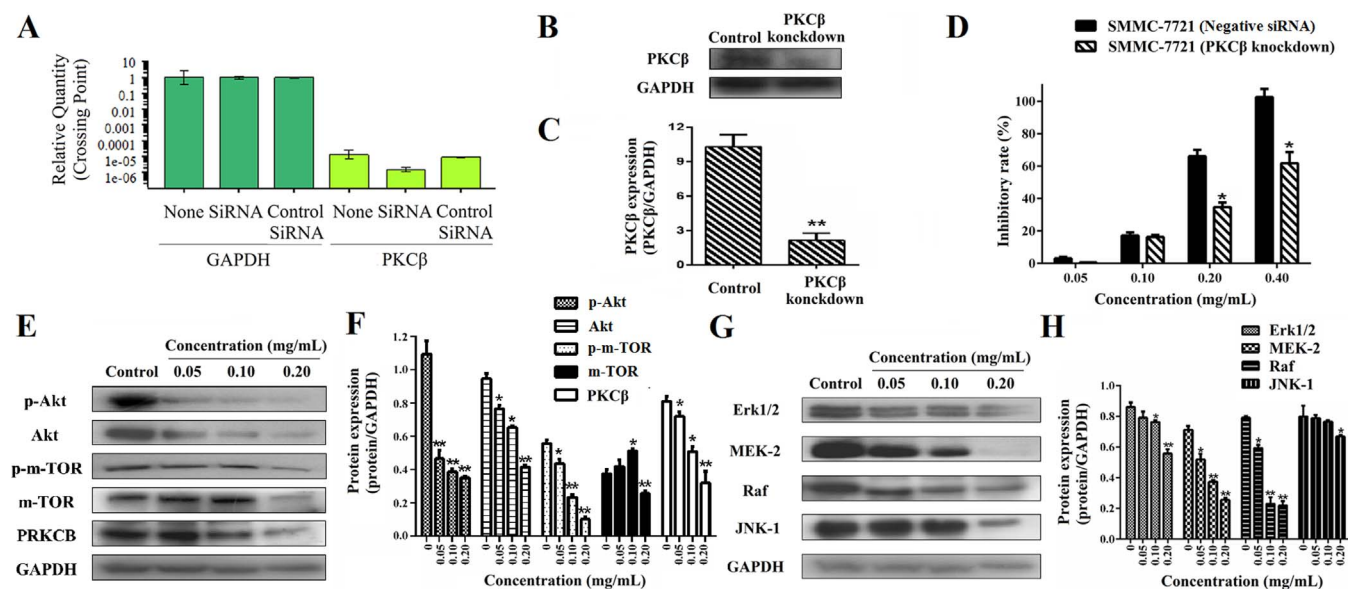


Figure 5 | *ESWE* inhibited cell growth and decreased protein expressions of Akt, P-Akt, m-TOR, P-m-TOR, PKC β , and Erk1/2, MEK-2, Raf and JNK-1 involved in cell growth. PKC β knockdown affects the *ESWE*'s anti-proliferation on SMMC-7721 cells. PKC β mRNA expression (A) and protein expression (B and C) in SMMC-7721 cells transfection with 80 nM PKC β siRNA using Lipofectamine 2000 reagent and SMMC-7721 cells (wild type) were determined by RT-PCR analysis and western blot assay. Full-length blots are presented in Supplementary Figure 5B. Transfection with a control siRNA construct served as a negative control. Data represents the means \pm SD ($n = 3$). * $p < 0.05$, ** $p < 0.01$ versus the PKC β expression in SMMC-7721 cells. (D) Effect of *ESWE* on cell proliferation in SMMC-7721 cells (wild type) and knockdown cells by MTT assay. After treated with PKC β siRNA for 24 h, cells were treated with *ESWE* for 48 h. Data represents the means \pm SD from three repeated experiments. Five wells were treated in each experiment. (E) Bands were corresponding to Akt, P-Akt, m-TOR, P-m-TOR, PKC β and GAPDH in SMMC-7721 cells. Full-length blots are presented in Supplementary Figure 5E. (F) Results were quantified by densitometry analysis of the bands form (E) and then normalization to GAPDH protein. (G) Bands were corresponding to Erk1/2, MEK-2, Raf, JNK-1 and GAPDH in SMMC-7721 cells. Full-length blots are presented in Supplementary Figure 5G. (H) Results were quantified by densitometry analysis of the bands form (G) and then normalization to GAPDH protein. Quantitation data showed *ESWE* decreased the proteins levels involved in cell growth in a dose-dependent manner compared to the untreated control. Values were expressed as means \pm SD ($n = 3$). * $p < 0.05$, ** $p < 0.01$, vs. control.

observed the inhibition on cell mobility by transwell system, and the knockdown of PKC β significantly reduced the inhibition on the migration of SMMC-7721 cells by *ESWE*, as compared with the control groups (Figure 6A and B). WB assay showed that MMP2, MMP9 and CXCR4 activity was strongly decreased, which dissected the alterations and function of MMPs and CXCR4 in *ESWE*-inhibited cell metastasis activities.

Knockdown of PKC β significantly reduced the inhibition on the proliferation and migration of SMMC-7721 cells by *ESWE*. So, the PLG, NF- κ B and P53 that are related to proteins cell proliferation and metastasis are also analyzed. PLG (Plasminogen) plays an essential role in the proteolytical degradation of extracellular matrix (ECM) and the basement membrane surrounding the primary tumor, which is a major determinant for cancer invasion and metastasis. Recent studies indicate that PLG affects adhesion, angiogenesis, differentiation and proliferation³⁰. NF- κ B is important and complicated in regulating cellular responses, and promotes cell proliferation and transformation, migration and initiation, promotion and progression of cancer^{31,32}. P53 gene is the most commonly mutated gene in human cancer, the normal gene product exerts the antiproliferative and antitransforming activity and in some cases promotes cell death via apoptosis³³. The above molecules are essential for migration, fusion, proliferation, and so on. Figure 6E showed PLG, NF κ B and P53 related to growth and metastasis were down-regulated by *ESWE*. In fact, there is an interrelation in the Erk1/2, MEK-2, Raf and JNK-1 of PLC γ /PKC, PI3K/AKT and MAPK signaling pathway^{32,34–36}. We, therefore, concluded that the inhibiting effect of *ESWE* on HCC was through synthetically regulating cell growth and metastasis signaling.

Taken together, results presented in this work demonstrate that *ESWE* inhibits HCC growth by downregulating the signaling of PKC β , Akt, m-TOR, Erk1/2, MEK-2, Raf and JNK-1 as well as phosphorylation of Akt and m-TOR, simultaneously increasing cyclin D1 protein levels and decreasing amount of cyclin E, cyclin B1 and cdc2 of the cycle proteins. Furthermore, *ESWE* reduced MMP2, MMP9 and CXCR4, and PLG, NF κ B and P53 activities. These results support the likelihood that *ESWE* affects HCC growth by blocking two cellular processes essential for tumor development: cell proliferation (including arrest cell cycle) and tumor metastasis. These data support the development of *ESWE* as a novel, multiple action growth and metastasis signaling inhibitor targeting the PKC, AKT, MAPK signaling and MMP2, MMP9 and CXCR4, and PLG, NF κ B and P53.

Methods

Reagents. RNase and PI were obtained from Sigma (USA). Akt rabbit mAb, Raf-1 rabbit mAb, JNK-1 rabbit mAb, MMP-2 rabbit mAb and MMP-9 rabbit mAb were obtained from Epitomics (USA). P-Akt, P-m-TOR, MEK 1/2 rabbit mAb and p44/42 MAPK (Erk1/2) rabbit mAb were purchased from Cell Signaling (USA). PKC β rabbit PolyAb, MTOR rabbit PolyAb, CXCR4 mouse mAb, NF- κ B rabbit PolyAb, PLG rabbit PolyAb, Cyclin B1 rabbit polyAb, cyclin D1 mouse mAb, cyclin E rabbit polyAb and cdc2 rabbit polyAb, HRP-conjugated GAPDH mAb were from proteintech group (USA). Mutated P53 rabbit mAb was from Abcam (England). Goat anti-rabbit IgG were purchased from Santa Cruz Biotechnology (CA, USA). Protease inhibitor cocktail was from Roche (Roche Tech., Switzerland). Rabbit anti-mouse IgG, goat anti-rabbit IgG, BCA protein assay reagent kit and enhanced chemiluminescent (ECL) plus reagent kit were obtained from Pierce (Pierce Biotech, USA). Total RNA extracted kit was from Fastagen (Fastagen, China). Revert AIDTM. first strand cDNA synthesis kit was from Fermentas (Hanover, Lithuania). RNAi was from Fermentas (Hanover, Lithuania). Other reagents used were analytical grade.

Animals and cell culture. 6–8 weeks ALB/C nude male mice (the Experimental Animal Center of Xi'an Jiaotong University, Xi'an, China) were used for all experiments. The methods were carried out in accordance with the approved

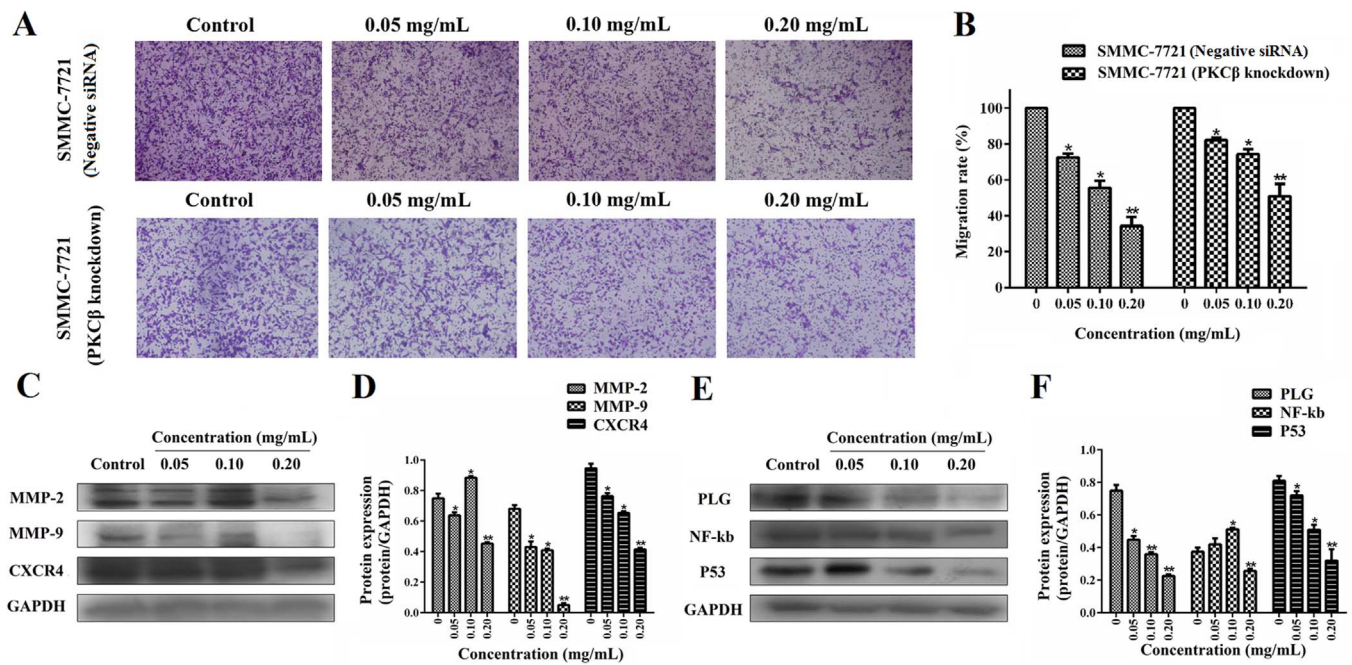


Figure 6 | Dose-response study of *ESWE* on the migration of cells transfected with siRNA of PKC β and normal SMMC-7721 cell *in vitro*. (A) Photographs of the knockdown cell and wide type cell migration through the polycarbonate membrane stained by 0.2% crystal violet. (B) Quantification of the number of cells (transfected with siRNAs of PKC β and wide type cells) migrating through the polycarbonate membrane. Data represents the means \pm SD from three repeated experiments. Five wells were treated in each experiment. (C) Bands were corresponding to MMP-2, MMP-9, CXCR4 and GAPDH in SMMC-7721 cells. Full-length blots are presented in Supplementary Figure 6C. (D) Results were quantified by densitometry analysis of the bands form (C) and then normalization to GAPDH protein. (E) Bands were corresponding to PLG, NF-kb, P53 and GAPDH in SMMC-7721 cells. Full-length blots are presented in Supplementary Figure 6E. (F) Results were quantified by densitometry analysis of the bands form (E) and then normalization to GAPDH protein. Quantitation data showed *ESWE* decreased the proteins levels involved in cell migration in a dose-dependent manner compared to the untreated control. Values are expressed as means \pm SD (n = 3). * $p < 0.05$, ** $p < 0.01$, vs. control.

guidelines of the regional authorities according to China animal-care regulations. SMMC-7721, BEL-7402 and Hep G2 cancer cells from Shanghai Institute of Cell Biology in the Chinese Academy of Sciences were maintained in RPMI1640 and MEM with 10% FBS.

Preparation of eupolyphaga sinensis walker extract (ESWE). The raw material used in the study was commercially available as dry matter. The raw material was crushed and soaked in 70% ethanol overnight and then refluxed gently in 10 volumes of 70% ethanol (v/w) for 1 h for two times. The merged extracting solutions were filtered, and supernatant was evaporated under reduced pressure and dried in vacuum conditions overnight. The dry powder was dissolved and stored at 4°C before use. In the next assays, ESWE was dissolved in serum free medium with DMSO as cosolvent, the equal DMSO in serum free medium was as control.

Cell proliferation assay. SMMC-7721, BEL-7402 and HepG2 cells (1×10^4) or siRNA cells of SMMC-7721 were cultured in 96-well plates, and fresh medium with or without *ESWE*, was added for 48 h. Cell proliferation reagent MTT was added and incubated at 37°C and 5%CO $_2$ for 4 h. Absorbance was then measured at 490 nm with microplate reader (Bio-RAD instruments, USA). In addition, SMMC-7721 cells were cultured in 6-well plates in fresh medium with or without *ESWE* for 5 days. The cell numbers were analyzed for the dynamic proliferation rate of cells. The experiment was repeated three times independently.

Colony formation assay. SMMC-7721, BEL-7402 and HepG2 cells were cultured in 6-well plates and fresh medium with or without *ESWE*, was added for 10 ~ 15 days. Colonies with cell numbers of >50 cells per colony were counted after staining with crystal violet solution. All the experiments were performed in triplicate wells in three independent experiments³⁷.

Subcutaneous xenografts in athymic mice and H/E staining. Tumors were induced in 4-week-old nude mice (male immune-deficient BALB/C nude mice) by subcutaneous flank inoculation of SMMC-7721 cells. Tumors were measured once every three days and tumor volumes (V) [(d $^2 \times L$)/2] were calculated from caliper measurements, where d and L are the shortest and the longest diameters, respectively. Mice were treated with *ESWE* at 400.0 mg/kg and vehicle alone by intragastric administration, the number of mice in each group was 4. After 10 days, mice weight and tumor volume were recorded. The tumors were fixed in 4% paraformaldehyde for immunohistochemical analysis. Tumor specimens were embedded into paraffin and the paraffin sections (4-mm thick) were used for H/E staining. The Institutional

Animal Care and Use Committee approved all of the procedures performed in this study.

Microarray analysis. The RNA for the microarray experiments was extracted from SMMC-7721 cells. Microarray experiments were performed in the whole human genome oligo microarray (Agilent Technologies, 4 \times 44K, CA, USA), which represents more than 41000 human genes and transcripts. It was used in this study to further systematically screen the differentially expressed genes between control and *ESWE*-treated cells at the Shanghai Biotechnology Corporation. Single stranded and double stranded cDNA was synthesized from total RNA samples (2 μ g) according to Agilent Gene-Chip Expression Analysis Technical Manual. The cRNA was purified and fluorochrome labeled with Cy3, and then fragmented and hybridized to the gene chip at 65°C with rotation for 17 h^{38,39}. Data were normalized by the Quantile method. The 2-fold change between two groups was the threshold for significant regulation. All microarray datasets were submitted to the "Gene Expression Omnibus" with an accession number of GSE52554.

Analyses of the cell cycle. SMMC-7721 cells were cultured in six-well culture plates for 24 h and incubated for 24 h after the medium was changed to serum-free medium. Cells were incubated for another 48 h with *ESWE* (0, 0.05, 0.10 and 0.20 mg/mL). The cell cycle was assessed according to the percentage of cells with DNA using the PI staining technique as described previously⁴⁰. Obtained data were analyzed with Modfit LT software.

siRNA analysis. Specific knockdown was achieved using siRNAs against PKC β or a control siRNA. A smart pool of double-stranded siRNA against PKC β as well as nonspecific siRNA was obtained from Shanghai GenePharma Co., Ltd. For transfection, siRNA was delivered at a final concentration of 50 nM using Lipofectamine 2000 reagent (Invitrogen) according to the manufacturer's instructions⁴¹. We incubated the cells for 24 h to allow knockdown of PKC β . These cells were used for proliferation and transwell assays⁴².

RNA isolation and RT-PCR. Total RNA of SMMC-7721 cells treated with or without *ESWE* were isolated using total RNA extracted kit. The total RNA was reversely transcribed in 20 μ l reaction solution using the Revert AIDTM first strand cDNA synthesis kit. Its integrity and subsequent RT-PCR performed for PKC β , PLG, IL3RA and GAPDH were described as previously. The sequence details of individual pairs of primers of PKC β , PLG, IL3RA and GAPDH were in Supplementary Table 1. The PCR reactions were performed with the Thermal Cycler Dice Real Time System (Takara,



Japan) in 96-well reaction plates. The relative amount of mRNA for each gene was normalized and represented as the ratio of the mRNA value of a target gene to that of the GAPDH gene.

Migration assay. A cell migration assay was performed using a transwell system, which allows cells to migrate throughout an 8- μ m pore size polycarbonate membrane of millicell[®]. Briefly, SMMC-7721 cells were first serum-starved for 24 h and then plated (1×10^4 cells/well) in serum-free medium containing ESWE at concentrations of 0.5, 1.0, 2.0 mg/mL in the upper chamber of a 12-well plate. The lower chamber was filled with 1.5 mL medium containing 10% FBS. After 48 h, cells remaining on the upper surface of the membrane were scraped using a cotton swab and the cells on the lower surface of the membrane were fixed with cold methanol for 15 min and stained with 0.2% crystal violet. Cells that had migrated to the bottom of the membrane were visualized and counted using an inverted microscope. For each repetition, cells in four randomly selected fields were counted and averaged. Data were expressed as a ratio to the untreated group.

Western blot analysis. The SMMC-7721 cells treated with or without ESWE for 48 h were prepared by extracting proteins with RIPA lysis buffer containing protease inhibitor cocktail and phosphatase inhibitor cocktail on ice. Cell lysates were analyzed for western blot analysis with primary antibodies, followed by enhanced chemiluminescence. Blots were reprobed with GAPDH to compare protein load in each lane⁴³.

Statistical analysis. Data were given as mean \pm S.D. in quantitative experiments. Statistical analyses of differences between the groups were performed with ANOVA by Student's *t* tests. A *p*-value less than 0.05 was considered statistically significant.

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Author contributions

L.C.H. designed the conception and reviewed the manuscript. Y.M.Z. performed the research, and wrote the manuscript. B.L.D. and W.N.M. performed development of methodology. J.P.Q. and R.L. acquired data (provided animals, provided facilities, etc.). Y.Z.Z. and D.D.Z. performed analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis).

Additional information

Supplementary information accompanies this paper at <http://www.nature.com/scientificreports>



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