Improved solubility, dissolution rate, and oral bioavailability of main biflavonoids from Selaginella doederleinii extract by amorphous solid dispersion

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ABSTRACT
Amentoflavone, robustaflavone, 2\(^{''}\)3\(^{''}\)-dihydro-3\(^{'''}\)-biapigenin, 3\(^{'''}\)-3\(^{'''}\)-binaringenin, and delicaflavone are five major hydrophobic components in the total biflavonoids extract from Selaginella doederleinii (TBESD) that display favorable anticancer properties. The purpose of this study was to develop a new oral delivery formulation to improve the solubilities, dissolution rates, and oral bioavailabilities of the main ingredients in TBESD by the solid dispersion technique. Solid dispersions of TBESD with various hydrophilic polymers were prepared, and different technologies were applied to select the suitable carrier and method. TBESD amorphous solid dispersion (TBESD-ASD) with polyvinylpyrrolidone K-30 was successfully prepared by the solvent evaporation method. The physicochemical properties of TBESD-ASD were investigated by scanning electron microscopy, differential scanning calorimetry, and Fourier-transform infrared spectroscopy. As a result, TBESD was found to be molecularly dispersed in the amorphous carrier. The solubilities and dissolution rates of all five ingredients in the TBESD-ASD were significantly increased (nearly 100% release), compared with raw TBESD. Meanwhile, TBESD-ASD showed good preservation stability for 3 months under accelerated conditions of 40 °C and 75% relative humidity. A subsequent pharmacokinetic study in rats revealed that \(C_{\text{max}}\) and \(AUC_{0-\text{t}}\) of all five components were significantly increased by the solid dispersion preparation. An in vivo study clearly revealed that compared to raw TBESD, a significant reduction in tumor size and microvascular density occurred after oral administration of TBESD-ASD to xenograft-bearing tumor mice. Collectively, the developed TBESD-ASD with the improved solubility, dissolution rates and oral bioavailabilities of the main ingredients could be a promising chemotherapeutic agent for cancer treatment.

Introduction

Selaginella doederleinii Hieron, a medicinal herb that is widely distributed in Southern China and Southeast Asia, has been traditionally used as a folk medicine for the treatment of various cancers, especially for nasopharyngeal carcinoma, lung cancer, and cervical cancer (Liu et al., 2011; Sui et al., 2016). Due to the anti-oxidation (Saroni Arwa et al., 2015; Li et al., 2017), anti-inflammatory (Kedi et al., 2018), anti-diabetic (Zheng et al., 2011), anti-virus (Coulerie et al., 2013), and anti-cancer effects (Li et al., 2014; Park & Kim, 2019). Phytochemical studies revealed that the chemical components of S. doederleinii mainly consist of biflavonoids, such as amentoflavone, robustaflavone, 2\(^{''}\)3\(^{''}\)-dihydro-3\(^{'''}\)-biapigenin, 3\(^{'''}\)-3\(^{'''}\)-binaringenin, and delicaflavone (Li et al., 2013; Yu et al., 2017). Modern pharmacological studies have also revealed that the ethanol extract of S. doederleinii could induce cancer cells apoptosis, significantly inhibit the tumor growth, and enhance the anti-tumor immune response. However, this extract did not display oral acute toxicity in vivo (Sui et al., 2016). Due to including the abundant biflavonoids, the total biflavonoids extract of S. doederleinii (TBESD) had more powerful anti-cancer activities than its ethanol extract (Yao et al., 2017). Although, TBESD has shown many advantages, its low aqueous solubility, low gastrointestinal permeability, and poor oral absorption and bioavailability of the biflavonoids in the...
extract posed problems in the drug delivery (Yang et al., 2016; Chen et al., 2018).

Drug delivery via the oral route is the most popular route owing to the safety, security, and simplicity of the administration. Investigations on the feasible oral drug delivery systems for water-insoluble drugs are still current administration. Investigations on the feasible oral drug technologies used to produce SDs are classified as melting, determination the drug-excipient molecular interaction. The main factor involved in the physical stability and release of TBESD, which primarily relies on optimizing suitable pharmaceutical carriers and technologies, is warranted. To overcome these issues, an optimal selection of excipients for TBESD amorphous solid dispersion (TBESD-ASD), which is the main factor involved in the physical stability and release behavior of the drug from the ASD, should be performed to determine the drug-excipient molecular interaction. The technologies used to produce SDs are classified as melting, solvent evaporation, and melting-solvent methods (Paudel et al., 2013; Borba et al., 2016; Weerapol et al., 2017). As high temperatures during the melting process may induce the degradation or decomposition of the biflavonoids in TBESD, solvent evaporation was employed to prepare TBESD-ASD (Mohammadi et al., 2014).

In the current study, an ASD formulation was developed to ameliorate the solubility and dissolution rate of five major ingredients in the TBESD. Different excipients were tested to select the optimum carrier for TBESD-ASD using the solvent evaporation method. Finally, the ASD of TBESD was successfully formulated with polyvinylpyrrolidone K-30 (PVP K-30), one of the most common polymers used in commercial ASD formulations. The solid-state characteristics, dissolution behavior, and accelerated conditions for the stability of TBESD-ASD were investigated. Further, the oral bioavailability and antitumor effect in vivo were evaluated using an LC-ESI-MS/MS method and A549 xenograft-bearing mice models.

Materials and methods

Materials

TBESD was obtained from S. doederleinii using the reported methods (Yao et al., 2017): 103.82 mg/g, 37.52 mg/g, 44.40 mg/g, 53.36 mg/g, and 35.12 mg/g of amentoflavone, robustaflavone, 2”,3”-dihydro-3’,5’-biapigenin, 3’,3’’-binaringenin, and delicaflavone were respectively used. Chrysin (purity ≥98%, internal standard, IS) was acquired from Shanghai Winherb Medical Technology Co., Ltd. (Shanghai, China). Reference standards for amentoflavone, robustaflavone, 2”,3”-dihydro-3’,5’-biapigenin, 3’,3’’-binaringenin, and delicaflavone (purity ≥98%) were isolated from S. doederleinii and their structures were fully elucidated by UV, MS, 1H NMR, and 13C NMR and confirmed by comparison to the literature (Li et al., 2014). The chemical structures of amentoflavone, robustaflavone, 2”,3”-dihydro-3’,5’-biapigenin, 3’,3’’-binaringenin, and delicaflavone are shown in Figure S1.

Poloxamer 188, PVP K-30, and polyethylene glycol 4000 and 6000 (PEG 4000 and PEG 6000) were purchased from Sigma-Aldrich Co. (St. Louis MO). Acetonitrile (HPLC-grade) was supplied from Merck (Darmstadt, Germany). Acetic acid (HPLC-grade) was supplied by Aladdin (Shanghai, China). Ethanol (analytical grade) was purchased from Sinopharm Chemical Reagent Co., Ltd (Shanghai, China). All other chemicals were of analytical-grade or pharmaceutical-grade and were used without further purification.

Sample preparation

S. doederleinii supplied by a local Chinese medical store in Fuzhou, China; the voucher specimens (no. 180708), and authenticated by professor Hong Yao (Department of Pharmaceutical Analysis, Fujian Medical University, Fuzhou, China), were stored in the Phytochemistry Laboratory.
TBESD was isolated from *S. doederleinii* using our previously described procedure (Yao et al., 2017). Briefly, after dried whole plants were pulverized into powder (20–40 mesh); the sample extracted at 85 °C for 2 h with an eightfold volume of 70% ethanol three times, and the resulting ethanol extracts were combined, transferred to a flask, and evaporated in a rotatory evaporator (Buchi, Flawil, Switzerland) to condense into a paste. The ethanol extract was extracted twice with petroleum ether and then was filtered. The residues were extracted twice with an eightfold volume of dichloromethane and were then filtered, and extracted twice with an eightfold volume of ethyl acetate and were filtered again. Ultimately, the ethyl acetate filtrate was concentrated using a rotary evaporator at 55 °C, and it was lyophilized to obtain TBESD.

Preparative fractionation of TBESD was performed on a 280 mL coil of 1.8 mm i.d. polytetrafluoroethylene and a 20 mL sample loop. The two-phase solvent system consisted of n-hexane-ethyl acetate–methanol–water 1:2:1.5:1.5. The coil column was first entirely filled with the upper phase; subsequently, the mobile lower phase was pumped (1 mL/min) into the head end of the inlet column. After reaching hydrodynamic equilibrium (850 rpm), the sample (500 mg) was dissolved in 20 mL of each two-phase solvent system and injected into the column through the injection valve.

**Content analysis of the main ingredients in TBESD**

The amount of amentoflavone, robustaflavone, 2′′,3′′-dihydro-3′,3′′-biapigenin, 3′,3′′-binaringenin, and delicaflavone were determined with a Shimadzu LC-20AD HPLC system equipped with column Ultimate® XB-C18 (100 × 4.6 mm, 3.5 μm; Welch Materials, Inc., Ellicott, MD). The mobile phase consisted of water (containing 0.05% acetic acid, A) and acetonitrile (B) and was used at a flow rate of 1 mL/min. Column temperature was maintained at 30 °C, and detection wavelength was 270 nm. The quantification method from our previous report was applied (Chen et al., 2019). The following gradient elution program was used: 0–4 min, 30–40% B; 4–23 min, 40–42% B; 23–25 min, 42–46% B.

**Partition coefficients**

The lipophilicity of the five major active ingredients in TBESD was evaluated by the shake-flask method, a standard procedure for testing the physicochemical property of chemicals (Organization for Economic Cooperation and Development (OECD), 2004; Ogden & Dorsey, 2019). The 1-octanol/water partition coefficients of the ingredients in TBESD were determined with a Shimadzu LC-20AD HPLC system equipped with column Ultimate® XB-C18 (100 × 4.6 mm, 3.5 μm; Welch Materials, Inc., Ellicott, MD). The mobile phase consisted of water (containing 0.05% acetic acid, A) and acetonitrile (B) and was used at a flow rate of 1 mL/min. The lipophilicity of the five major active ingredients in TBESD were determined with a Shimadzu LC-20AD HPLC system equipped with column Ultimate® XB-C18 (100 × 4.6 mm, 3.5 μm; Welch Materials, Inc., Ellicott, MD). The mobile phase consisted of water (containing 0.05% acetic acid, A) and acetonitrile (B) and was used at a flow rate of 1 mL/min. Column temperature was maintained at 30 °C, and detection wavelength was 270 nm. The quantification method from our previous report was applied (Chen et al., 2019). The following gradient elution program was used: 0–4 min, 30–40% B; 4–23 min, 40–42% B; 23–25 min, 42–46% B.

**Partition coefficients**

The amount of amentoflavone, robustaflavone, 2′′,3′′-dihydro-3′,3′′-biapigenin, 3′,3′′-binaringenin, and delicaflavone were determined by HPLC. The partition coefficients of five biflavonoids in TBESD were calculated using the following equation:

\[
\log K_{ow} = \log C_o/C_w
\]

where \(C_o\) is the concentration of biflavonoids in the 1-octanol phase at equilibrium; \(C_w\) is the concentration of biflavonoids in the aqueous phase at equilibrium; \(K_{ow}\) is the 1-octanol/water partition coefficients. All experiments were carried out at least in triplicate.

**Preparation of TBESD-ASD**

A solid dispersion of TBESD with various polymers (Poloxamer 188, PVP K-30, PEG 4000, and PEG 6000) was prepared by the solvent method. The ratios of TBESD/polymer (w/w) are shown in Table 1. Briefly, the prescribed amounts of TBESD and polymer were separately dissolved in a certain volume of ethanol. The two solutions were mixed with ultrasonic stirring for 20 min, and then evaporated at 60 °C under vacuum to remove the solvent. The residue was evaporated to dryness under reduced pressure at 50 °C for 12 h. The dried samples were crushed and screened through a mesh size of 80. Prepared solid dispersions were kept in a desiccator until further investigation.

Several elements were considered during the preparation process for the TBESD-ASD. According to preliminary studies, the main variables that affect the solubility were ethanol concentration (%), mesh level (mesh), ultrasonic time (min), and drying temperature (°C) (labeled as A, B, C, and D in Table 2). In this study, an L9 (3⁴) orthogonal test design was employed to further optimize the preparation conditions for TBESD-ASD. The orthogonal design scheme was composed of four-factors and three-levels, totaling nine experimental groups, which is very effective and economical (Gao et al., 2012; Chen et al., 2019). The desirability function for the solubility of ASD was at the maximum level and was thus selected to optimize the TBESD-ASD formulation. In the current research, all data were determined as the mean of triplicate samples.

**Table 1. Formulation of TBESD solid dispersions.**

| Ratio | TBESD (mg) | Polymer (mg) |
|-------|------------|--------------|
| 1:3   | 20         | 60           |
| 1:4   | 20         | 80           |
| 1:5   | 20         | 100          |
| 1:10  | 20         | 200          |

**Table 2. The factors and levels of orthogonal test.**

| A  | B  | C  | D  |
|----|----|----|----|
| 1  | 100| 100| 20 | 55 |
| 2  | 95 | 80 | 15 | 50 |
| 3  | 80 | 65 | 10 | 45 |

The amount of TBESD at 20 mg.
Characterization of TBESD-ASD

Morphology
Scanning electron microscopy (SEM) analysis of the TBESD and TBESD-ASD was performed using a Hitachi SU8010 microscope (Hitachi, Tokyo, Japan). The samples were attached to an aluminum sample holder using a double-sided adhesive tape.

Differential scanning calorimetry
Differential scanning calorimetry (DSC) measurements were performed to examine the solid-state characteristics of TBESD, PVP K-30, PMs, and TBESD-ASD. Thermal analysis was carried out with STA 449 F3 Jupiter® (Netzsch, Selb, Germany). Samples were placed inside a crimped aluminum pan maintained at 25°C for 5 min, and then heated to 300°C at a heating rate of 10°C/min.

Powder X-ray diffraction (PXRD)
The crystalline state of TBESD, PVP K-30, PMs, and TBESD-ASD was evaluated using powder X-ray diffraction (DYS261/ Xpert3, CEM, Matthews, NC). The samples were collected with a scan rate of 4°/min⁻¹ in an angular range from 5° to 70° (2θ) using Cu Kα radiation (λ = 1.54 Å, wavelength) and operating at 40 kV and 40 mA.

FT-IR spectroscopy
Infrared analysis of the solid complexes was carried out on a Nicolet iS5 (Thermo-Nicolet Instrument Co., Waltham, MA) at resolution of 4 cm⁻¹ over the range, 4000–500 cm⁻¹. The FT-IR spectrograms of TBESD, PVP K-30, PMs, and TBESD-ASD were determined and compared.

Solubility study
To evaluate the solubility of the ingredients in the ASD of TBESD with various polymers, an excess amount of ASD was added to 1 mL of distilled water. The effect of pH on the solubility of the ingredients in the ASD of TBESD with PVP K-30 was determined in 1 mL of PH 1.2, 4.5, and 6.8 buffers. In all cases, samples were placed in a thermostat oscillator and shaken for 48 h at 37°C, and then centrifuged at 12,000×g for 15 min at 37°C. After appropriate dilutions, the supernatants were subjected to HPLC. The experiments were carried out in triplicate.

In vitro dissolution study
The dissolution studies were performed with a dissolution test apparatus III (ZRS-8GD, Tianjin, China) at a paddle speed of 100 rpm in 200 mL of 6.8 phosphate buffer maintained at 37 ± 0.5°C (Chinese Pharmacopoeia Commission, 2015). Each formulation equivalent to 300 mg of TBESD was added to a dissolution vial. At predetermined time intervals (5, 10, 20, 30, 45, 60, 90, 120, and 180 min), 2 mL sample solution was withdrawn, and an equal volume of fresh medium was replenished. The sample solution was centrifuged at 12,000×g for 5 min at 37°C and the supernatants were subjected to HPLC.

Stability study
The sample powder was stored in open transparent glass containers. The chemical stability test for the TBESD-ASD at 4500 ± 500 Lx illumination was carried out for 10 days to investigate the stability of the five components of TBESD in a strong light exposure environment.

The chemical stability test for the TBESD-ASD at 60°C was carried out for 10 days to investigate the stability of the five components of TBESD in a high temperature environment.

The chemical stability test for the TBESD-ASD under accelerated (40 ± 2°C/75 ± 5% RH) condition was carried out for 3 months to evaluate the preservation stability. The sample powder was enclosed into capsules in tightly capped brown glass vials to protect it from the light. At predetermined time intervals, sample powder was completely dissolved in ethanol and the five components content in TBESD-ASD were analyzed by HPLC.

In vivo study

Animals
All animal experiments were approved by the Institutional Animal Care and Use Committee of Fujian Medical University. Animal welfare and experimental procedures were carried out following an approved protocol from the Guide for the Animals Care and the Ethics Committee of Fujian Medical University. Eight-week-old Sprague-Dawley (SD) male rats (n = 12, 250 ± 20 g) were provided by the Laboratory Animal Center of Fujian Medical University. Six-week old BALB/c male nude mice (n = 36, 20 ± 2 g) were obtained from National Rodent Laboratory Animal Resource (Shanghai, China) and bred in the Laboratory Animal Center of Fujian Medical University. All rats were allowed to acclimatize to the laboratory conditions, including an ambient temperature of 25 ± 2°C, a 12-h light/dark cycle, and an RH of 55 ± 5% over 1 week before the experiments. Rats were granted free access to food and water, and fasted with free access to water for 12 h before drug administration.

Pharmacokinetic study
In this experiment, 12 male SD rats were randomly and equally divided into the TBESD group and a TBESD-ASD group (n = 6). Thereafter, both groups were orally administered drugs that were equivalent to 20.76, 7.50, 8.88, 10.67, and 7.02 mg/kg for amentoflavone, robustaflavone, 2′,3′-dihydro-3′,3′-biapigenin, 3′,3′-binaringenin, and delicaflavone, respectively (200 mg/kg for TBESD). After
administration, 200 μL of blood samples was obtained from
the tail vein at 0, 3, 8, 15, 30, 45, 60, 90, 120, 240, 360, 480,
720, and 1440 min. All blood samples were immediately cen-
trifuged at 3000×g for 10 min at 4 °C. Thereafter, the super-
natant was retrieved and stored at −80 °C until analysis.

Plasma samples were treated by the methanol deprotein-
ization method and the plasma concentration of amentoflavo-
ne, robustaflavone, 2′,3′-dihydro-3′,3′″-biapigenin, 3′,3″-
binaringenin, and delicaflavone was analyzed using a previ-
ously developed LC-ESI-MS/MS method (Chen et al., 2018).

A highly sensitive and credible LC-ESI-MS/MS method was
developed for the PK comparative study of TBESD and
TBESD-ASD with simultaneous quantification of the five main
biflavonoids in rat plasma. The pharmacokinetic parameters
for free TBESD and TBESD-ASD were calculated by the non-
compartamental method using the DAS pharmacokinetic soft-
ware Version 3.0 (Bontz Inc., Beijing, China).

Antitumor efficacy study
The xenograft-bearing mice model of A549 tumor was estab-
lished by injecting of 1.0×10⁷ A549 cells via s.c. into the
right front armpit area of BALB/c male athymic nude mice. Mice
were randomly and equally divided into the vehicle control,
TBESD, and TBESD-ASD groups when the xenograft
tumors were palpable and had an average size of
80–100 mm³ (n = 6). The vehicle, TBESD (200 mg/kg), and
TBESD-ASD groups (200 mg/kg) were administered p.o. every
day for 16 days. The positive group was administered 2 mg/
kg doxorubicin (Dox) i.v. once every three days via the tail-
vein until sacrifice. Tumor size and body weight were meas-
ured and recorded every two days.

Tumor volume (V) was calculated using the formula:
V=WL²/2, where width (W) and length (L) were measured
with an electronic caliper. Mice were killed one day following
the last treatment, and tumors were collected, weighted, and
photographed. The tumor inhibition effect was calculated
using the following equation: tumor suppression (%)=(1 − T/
C)×100 where T represents the average tumor weight of the
treated group and C represents that of the control group.

Immunohistochemistry (IHC) was carried out to further
validate the antitumor efficacy of free TBESD and TBESD in
the solid dispersion formulation in vivo. Tumors were col-
clected and fixed in 4% neutral formalin for 24 h, and then
embedded in paraffin. For the assessment of tumor micro-
vesSEL density (MVD), some sections were deparaffinized
and rehydrated. After quenching the endogenous peroxidase
activity and blocking nonspecific binding sites, the slides
were co-cultured with primary monoclonal CD34 antibody.
For the quantification of positively stained vessels, the num-
ber of microvessels was counted in three randomly selected
high-power fields by Image pro-plus software Version 6.0 at
200× magnification.

Statistical analysis
All data are expressed as mean ± SD. Significance differences
were derived by Student’s t-test. *p<.05 was considered to
indicate statistical significance while **p<.01 was considered
to indicate a highly significant difference.

Results and discussion
Partition coefficients
Before employing the formulation strategies to improve the
solubility of poorly soluble drugs, the lipophilicity of the
biflavonoids in TBESD should be considered. To address the
first objective of this study, we measured the log Kow of five
biflavonoids of TBESD in the normal internal environment pH
buffers and compared our measurements to the predicted
values of log p values obtained from Molinspiration Property
Calculator (Molinspiration Cheminformatics, Bratislava, Slovak
Republic) (Lipinski et al., 2001).

The lipophilicity is an important physiochemical property
for characterizing the absorption, distribution, metabolism,
and excretion (ADME) behavior of a drug. The results
obtained for the five biflavonoids by the shake-flask method
aligned well with the predicted values (Supplementary data,
Table S1). However, their high Lipophilicity, with log p values
ranging from 3.5 to 4.5, limit their absorption and bioavail-
ability, as well as clinical utility. These findings suggest that a
suitable formulation strategy must be developed to enhance
the solubility and bioavailability of the main active ingre-
dients in TBESD.

Preparation of TBESD-ASD
Similar to general pharmaceutics, the development of solid
dispersions relies on having access to suitable pharmaceut-
tical technologies and carriers to prepare the formulation (Yu
et al., 2018). Previously, we demonstrated that the solvent
evaporation method using anhydrous ethanol as a solvent
enabled the successful development of TBESD-ASD.
Depending on the properties of the carriers, different
molecular weights and surface activity polymers have been
reported to improve the solubility and concomitantly the dis-
solution rate of the hydrophobic drug in the solid dispersion
(Weerapol et al., 2017). In this study, different polymers were
evaluated to determine the optimal carrier for enhancing the
solubility of all five components of TBESD when formulated
as an ASD. All five components achieved the highest solubil-
ity among the screened polymers when the TBESD solid dis-
persion was formed with PVP K-30 (Figure 1). The enhanced
solubility of amentoflavone, robustaflavone, 2′,3′-dihydro-
3′,3″-biapigenin, 3′,3″-binaringenin, and delicaflavone owing
to solid dispersion with PVP K-30 was 16.98-, 17.47-, 17.59-,
18.42-, and 19.48-fold higher than those of the biflavonoids
for raw TBESD in water, respectively (Table 3). In addition,
the nonhomogeneous and sticky mass solid dispersions were
obtained by using Poloxamer 188, PEG 4000, and PEG 6000
as the carriers, might be due to the formation of a eutectic
or monotectic mixture by the drug and polymer (Vippagunta
et al., 2007; Brough and Williams, 2013). Altogether, it can be
assumed that among the screened carriers, PVP K-30 exhib-
ited better mobility, miscibility, and more similarity in the
Figure 1. Solubility of the components of (A) TBESD after forming ASD with various polymers in distilled water at room temperature; Dissolution profiles of (B) amentoflavone, (C) robustaflavone, (D) 2',3'-dihydro-3',3'-biapigenin, (E) 3',3'-binaringenin, and (F) delicaflavone from TBESD and solid dispersion formulations, respectively (mean ± SD, n = 3).

Table 3. Solubility from TBESD and ASDs with PVP K-30 in various pH medium at room temperature (mean ± SD, n = 3).

| Components | Amentoflavone | Robustaflavone | 2',3'-Dihydro-3',3'-biapigenin | 3',3'-Binaringenin | Delicaflavone |
|------------|---------------|----------------|-------------------------------|--------------------|--------------|
| Pure extract in water | 39.96 ± 2.46 | 15.37 ± 4.61 | 16.44 ± 3.47 | 18.71 ± 3.26 | 9.65 ± 1.03 |
| ASDs in pH 1.2 | 434.15 ± 24.91 | 141.94 ± 17.15 | 178.65 ± 16.71 | 192.55 ± 21.46 | 112.54 ± 11.87 |
| ASDs in pH 4.5 | 468.49 ± 27.16 | 168.39 ± 13.84 | 204.21 ± 19.46 | 243.67 ± 23.16 | 134.79 ± 10.13 |
| ASDs in pH 6.8 | 615.69 ± 34.49 | 207.65 ± 21.89 | 246.32 ± 20.48 | 289.56 ± 28.13 | 175.16 ± 9.68 |
| ASDs in water | 678.38 ± 36.45 | 268.48 ± 31.73 | 289.16 ± 19.21 | 346.54 ± 14.56 | 187.98 ± 21.18 |
intermolecular interactions with the five biflavonoids. Hence, PVP K-30 was selected as the optimal polymers to form the TBESD-ASD for a further study.

Particle size reduction or generation of the amorphous states which could be significantly influenced on the surface area and solubility has attracted considerable attention (Onoue et al., 2011). Several factors influence the solubility of the ASD in the preparation process. In the current research, the preparation conditions of the ASD, including the ethanol concentration (%), mesh level (mesh), ultrasonic time (min), and drying temperature (°C), were optimized according to the orthogonal experiment design of L9 (3^4). To obtain high-quality TBESD-ASD with high solubility and dispersibility and successful enhancement of the solubility of the biflavonoids in TBESD, the solid dispersion technique provided better wettability and dispersibility in TBESD-ASD. These results suggest that there was a crystal transition of TBESD from the crystalline to the amorphous state in the SD.

### Characterization of TBESD-ASD

**Morphology characteristics**

The SEM micrographs of initial substances and TBESD-ASD exhibited clear morphological changes for the powder particles after ASD formation. Figure 2(A–C) shows the micrographs of TBESD, PVP K-30, and TBESD-ASD. The SEM image from the initial TBESD revealed an irregular shape with a rough surface. In contrast, the original PVP K-30 exhibited the typical spherical shape with a smooth surface. The TBESD-ASD also displayed the characteristic smooth surface. These results demonstrated that the formation of an ASD system containing TBESD resulted in a homogeneous dispersion of TBESD into the polymeric carrier.

**FT-IR spectroscopy**

The infrared spectra of PVP K-30, the PMs, TBESD-ASD, and TBESD are shown in Figure 3(B). The presence of PVP K-30 was confirmed by the appearance of a peak at 2157.0 cm⁻¹ in the IR spectrum of the PMs, which corresponds to the C=O stretching of the pyrrolidone functional group unique to PVP K-30. However, the pyrrolidone peak in the TBESD-ASD was shifted slightly from 1657.56 cm⁻¹ observed in the ASD’s spectrum due to the hydrogen bonded complex formation between PVP K-30 and biflavonoids in TBESD.

**Solubility study**

The compounds in TBESD, including amentoflavone, robustaflavone, 2',3',3'-dihydro-3',3'-biapigenin, 3',3''-binaringenin, and delicaflavone, were analyzed by HPLC. As illustrated in Table 3, the solubility of the five major ingredients in pure TBESD was extremely low in water. However, all five ingredients in TBESD-ASD exhibited excellent solubility compared to free TBESD when formulated as an ASD with PVP K-30. The solid dispersion technique provided better wettability and dispersibility and successful enhancement of the solubility of the biflavonoids in TBESD. As is shown in Table 3, the compound solubility from TBESD-ASD displayed a profound increase with an increase in pH. Solubility of amentoflavone was 1.56-fold higher at neutral environments than the solubility found at pH 1.2. In contrast, robustaflavone, 2''',3'''-dihydro-3',3'''-biapigenin, 3',3'''-binaringenin, and delicaflavone respectively exhibited 1.89-, 1.62-, 1.80-, and 1.67-fold higher solubility. Such a significant increase in TBESD components’ solubility, might be attributed to the changed glass transition temperature owing to the presence of hydrogen bonded network between PVP K-30 and TBESD (Roos et al., 2003).

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**Table 4. Results of the orthogonal experiment (n = 3).**

| A | B | C | D | EE (%) |
|---|---|---|---|-------|
| 1 | 1 | 1 | 1 | 0.806 |
| 2 | 1 | 2 | 2 | 0.840 |
| 3 | 1 | 3 | 3 | 0.814 |
| 4 | 2 | 1 | 2 | 0.803 |
| 5 | 2 | 3 | 1 | 0.795 |
| 6 | 2 | 3 | 1 | 0.799 |
| 7 | 3 | 1 | 3 | 0.852 |
| 8 | 3 | 2 | 1 | 0.810 |
| 9 | 3 | 3 | 2 | 0.540 |

K1, K2, and K3 are the sum scores of level 1, level 2, and level 3 for each factor. k1, k2, and k3 are the average scores of level 1, level 2, and level 3 for each factor. R is the range among the average scores for each factor, estimated by the difference between the highest and the lowest score average (R = k_max – k_min).

**DSC results**

DSC was employed in order to confirm the solid state of TBESD, PVP K-30, the physical mixtures (PMs) and TBESD-ASD. As shown in Figure 2(D), the DSC thermograms of TBESD displayed a broad endothermic peak at 40–150°C, with an endothermic peak at 86.50°C. PVP K-30 and PM (TBESD:PVP K-30 = 1:5) also exhibited an endothermic peak around 63.50°C. However, the DSC curves of TBESD-ASD displayed a shift in the endothermic peak to a slightly higher temperature of 67.57°C, as a TBESD peak was not observed, this indicates that TBESD was converted to an amorphous state and was thus dispersed in the molecular form in PVP K-30.

**Powder X-ray diffraction**

X-ray diffractograms of TBESD, PVP K-30, PMs, and TBESD-ASD are shown in Figure 3(A). The PXRD pattern of TBESD showed a distinct peak at 2θ degree of 23.25, demonstrating the crystalline nature of TBESD. The characteristic crystalline peak observed in the diffraction PM patterns was missing in TBESD-ASD. These results suggest that there was a crystal transition of TBESD from the crystalline to the amorphous state in the SD.
Therefore, TBESD-ASD can be enclosed into intestine-dissolved capsules which can be released and absorbed in a nearly neutral intestinal environment.

**In vitro dissolution study**

The dissolution profiles of TBESD and TBESD-ASD were analyzed and the results are presented in Figure 1(B–F). Due to the low solubility of the five major biflavonoids in a pH 6.8 buffer, there was less than 25% release after 3 h. The cumulative amount of amentoflavone, robustaflavone, 2′,3′-dihydro-3′,3″-biapigenin, 3′,3″-binaringenin, and delicaflavone in the control group was 24.31%, 19.80%, 17.21%, 19.13%, and 16.35%, respectively.

The release rate of all five ingredients in TBESD-ASD was significantly higher than those in raw TBESD. After 60 min for the dissolution test, the release amount for amentoflavone, robustaflavone, 2′,3′-dihydro-3′,3″-biapigenin, 3′,3″-binaringenin, and delicaflavone released 86.91%, 87.29%, 83.66%, 85.79%, and 79.43%, respectively. At the end of the dissolution test, the release percentage for the five biflavonoids was 94.72%, 94.26%, 93.64%, 93.07%, and 91.22%, respectively. It was regarded as a full release and was significantly higher than that of TBESD. All results indicated that much faster and higher dissolution rates were obtained by TBESD-ASD compared with TBESD.

**Stability study**

The presence of numerous ingredients in the herbal extract increases the risk of interactions among the components as well as degradation due to exterior conditions such as light, heat, and humidity. Therefore, the chemical stability of the herbal extract in the exterior environment should be investigated periodically.

In our preliminary experiment, the biflavonoids of the *S. doederleinii* extract displayed stability issues in the aqueous environment. The stability test for TBESD-ASD was carried out for 10 days and the results are shown in Figure 4. The content of the ingredients in TBESD-ASD was monitored in 4500 ± 500 Lx illumination, 60°C and 92.5% RH conditions. Among the five ingredients in TBESD-ASD, 3′,3″-binaringenin and delicaflavone were especially unstable in a high temperature environment. In addition, the five biflavonoids of TBESD in ASD showed a relatively lower content than the ASD at 0 h in a strong light exposure environment. No significant difference was found for the loss of biflavonoids from the TBESD-ASD in the high humidity environment. This might be because the five major ingredients are flavonoids with potent anti-oxidative effects. The stability of TBESD-ASD enclosed in the capsules was investigated under accelerated (40 ± 2°C/75 ± 5% RH) conditions. As a result, no significant change in the content of all five biflavonoids in TBESD-ASD.

![Figure 2](image_url)
was found throughout the 90-day period (Supplementary data, Table S2). The solid dispersion technique can provide physical or steric hindrance, thereby preventing and reducing the rate of degradation of the active ingredients (Yu et al., 2017).

**In vivo study**

**Pharmacokinetic study**
A comparative pharmacokinetic study was carried out in rats for the oral administration of raw TBESD and TBESD-ASD (200 mg/kg body weight as TBESD). Thereafter, the plasma concentrations of the five biflavonoids were determined with a validated LC-ESI-MS/MS method. The plasma concentration–time curves for the studies ingredients in TBESD and TBESD-ASD are depicted in Figure 5, and the main PK parameters of the five biflavonoids are shown in Table 5. The plasma concentrations of the five active ingredients were significantly higher following oral administration of TBESD-ASD than TBESD. By comparing the PK parameters between TBESD-ASD and raw TBESD, we found that the mean $C_{\text{max}}$ and AUC$_{0-t}$ of amentoflavone, robustaflavone, 2",3"-dihydro-3',3"'-biapigenin, 3',3"'-binaringenin, and delicaflavone in TBESD-ASD group were 3.43/2.78, 2.15/1.99, 3.97/3.72, 5.38/5.14, and 8.68/8.43, respectively.

**Figure 3.** (A) PXRD patterns of TBESD, PVP K-30, PMs, and TBESD-ASD; (B) FT-IR spectra of TBESD, PVP K-30, PMs, and TBESD-ASD.
4.17/4.87, and 2.38/1.84-fold higher than those in the TBESD group, respectively. Such finding indicates that the absorption of the five active ingredients in the solid dispersion formulation substantially increased. Compared to the oral administration of TBESD, the MRT\(_{0-\infty}\) values of the five major active ingredients in the TBESD-ASD group were higher while their CL values were lower. The results indicated that the solid dispersion formulation increased blood retention times and decreased the elimination rates of the five biflavonoids from *S. doederleinii* (Yadav et al., 2019). Compared to the oral administration of TBESD, the relative bioavailability (RA) of amentoflavone, robustaflavone, 2"",3""-dihydro-3",3""-biapigenin, 3",3""-binaringenin, and delicaflavone from TBESD-ASD was 293%, 372%, 375%, 484%, and 278%, respectively. Thus, ASD was confirmed to be an effective oral formulation for TBESD formulation.

**Antitumor efficacy study**

The therapeutic efficacy of TBESD-ASD was evaluated in an A549 xenograft-bearing mice model, and treatment was initiated after tumors were fully established (when the tumor volume achieved an average size of 80–100 mm\(^3\)). Compared to the unrelenting growth in the control group, tumor volumes of mice in all treatment groups were significantly decreased (*p* < .01). As shown in Figure 6(B), both the TBESD-ASD and Dox displayed superior antitumor effect relative to free TBESD and saline. As expected, treatment with Dox was effective at suppressing the tumor and this was accompanied by a significant loss in body weight. Mice were killed within 24 h after the last gavage, and their tumor tissues were collected and weighed (Figure 6(A)). The tumor growth inhibition (TGI) rates for the TBESD, TBESD-ASD, and Dox groups were 29.48%, 46.00%, and 58.44%, respectively.

**Figure 4.** Chemical stability test of TBESD-ASD in difference exterior environment: (A) amentoflavone, (B) robustaflavone, (C) 2",3""-dihydro-3",3""-biapigenin, (D) 3",3""-binaringenin, and (E) delicaflavone (mean ± SD, *n* = 3).
In particular, there was no significant difference in body weight between the TBESD-ASD treatment group and the TBESD treatment group. To further detect the antitumor effects of the TBESD solid dispersion formulation, the level of tumor angiogenesis blocking in vivo was analyzed via IHC of CD34. The control group displayed a high tumor MVD of 96.24 ± 6.91 cells/mm². As shown in Figure 6(D) and Table S3, TBESD, TBESD-ASD, and Dox decreased the MVD counts of the xenografts tumor by 24.30%, 52.20%, and 59.91%, respectively. According to the MVD counts, the anti-tumor and anti-angiogenesis effects of TBESD-ASD were higher than those of free TBESD. Thus, we concluded that the TBESD-ASD is a promising formulation for exerting the therapeutic benefits of the bioactive ingredients in TBESD.

Table 5. Pharmacokinetic parameters of five active ingredients in rats following single oral administration of TBESD and solid dispersion formulations, respectively (mean ± SD, n = 6).

| PK parameters | Formulation | Amentoflavone | Robustaflavone | 2″,3″-dihydro-3′,3‴-biapigenin | 3′,3‴-binaringenin | Delicaflavone |
|---------------|-------------|---------------|---------------|-----------------------------|------------------|---------------|
| $K_e$ (h⁻¹)   | TBESD       | 1.31 ± 0.49   | 1.79 ± 0.40   | 1.23 ± 1.56                 | 1.44 ± 0.59      | 0.37 ± 0.20   |
|               | TBESD-ASDs  | 1.15 ± 0.77   | 4.37 ± 5.72   | 0.55 ± 0.09$^a$             | 1.04 ± 0.69      | 0.38 ± 0.64   |
| $t_{1/2}$ (h) | TBESD       | 1.00 ± 0.43   | 0.40 ± 0.09   | 1.18 ± 0.83                 | 0.55 ± 0.20      | 1.06 ± 0.13   |
|               | TBESD-ASDs  | 1.09 ± 0.43   | 0.45 ± 0.41   | 1.30 ± 0.27$^a$             | 0.74 ± 0.20      | 1.08 ± 0.37   |
| $T_{max}$     | TBESD       | 0.23 ± 0.05   | 0.25 ± 0.15   | 0.33 ± 0.13                 | 0.27 ± 0.12      | 0.22 ± 0.06   |
|               | TBESD-ASDs  | 0.33 ± 0.13   | 0.22 ± 0.06   | 0.29 ± 0.10                 | 0.23 ± 0.05      | 0.29 ± 0.10   |
| $C_{max}$     | TBESD       | 20.15 ± 13.35 | 18.09 ± 6.91  | 9.21 ± 3.93                 | 7.48 ± 4.68      | 13.61 ± 5.81  |
|               | TBESD-ASDs  | 69.13 ± 37.63 | 38.87 ± 16.24$^a$ | 36.16 ± 32.27$^b$ | 31.16 ± 25.82$^a$ | 32.34 ± 33.36$^a$ |
| $AUC_0-t$ (h·µg/mL) | TBESD | 14.83 ± 6.86 | 10.85 ± 5.41 | 9.52 ± 1.75 | 4.16 ± 2.28 | 18.08 ± 2.62 |
|               | TBESD-ASDs  | 41.30 ± 14.10$^a$ | 21.59 ± 6.61$^a$ | 35.43 ± 20.94$^a$ | 20.26 ± 13.63$^a$ | 33.26 ± 19.80$^a$ |
| $AUC_0-\infty$ (h·µg/mL) | TBESD | 15.13 ± 6.68 | 11.66 ± 5.75 | 9.98 ± 1.62 | 5.75 ± 2.55 | 24.81 ± 9.30 |
|               | TBESD-ASDs  | 44.47 ± 13.14$^a$ | 28.75 ± 13.00$^a$ | 39.43 ± 20.05$^a$ | 24.53 ± 21.93$^a$ | 65.35 ± 47.41$^a$ |
| $Cl$ (L/h)    | TBESD       | 1530.83 ± 447.31 | 739.59 ± 257.35 | 909.15 ± 146.41 | 2170.42 ± 895.12 | 307.28 ± 78.42 |
|               | TBESD-ASDs  | 507.81 ± 171.52$^a$ | 323.06 ± 192.84$^a$ | 289.20 ± 162.37$^a$ | 836.85 ± 696.73$^a$ | 238.59 ± 320.60 |
| $MRT_{0-\infty}$ (h) | TBESD | 1.09 ± 0.29 | 0.61 ± 0.04 | 1.33 ± 0.72 | 0.52 ± 0.07 | 1.76 ± 0.44 |
|               | TBESD-ASDs  | 1.15 ± 0.53  | 0.53 ± 0.08  | 2.18 ± 0.80  | 0.89 ± 0.29$^a$  | 1.81 ± 0.95  |
| $RF$          | TBESD       | 2.93 ± 0.72   | 3.72 ± 4.01   | 3.75 ± 5.46   | 4.84 ± 2.78      | 2.78 ± 1.46   |

$^a$Indicates significant difference at $p < 0.05$. $^b$Indicates significant difference at $p < 0.01$. (Table 6). In particular, there was no significant difference in body weight between the TBESD-ASD treatment group and the TBESD treatment group. To further detect the antitumor effects of the TBESD solid dispersion formulation, the level of tumor angiogenesis blocking in vivo was analyzed via IHC of CD34. The control group displayed a high tumor MVD of 96.24 ± 6.91 cells/mm². As shown in Figure 6(D) and Table S3,
Figure 6. *In vivo* therapeutic study and Immunohistochemistry study of different TBESD formulations in a mice model. (A) Xenograft tumor in each group of mice after treatment, (B) xenograft tumor’s growth curve of mice (mean ± SD, n = 6), (C) the average weight change of mice (mean ± SD, n = 6), and (D) representative CD34 staining (×200) of xenograft tumor in each group.
Table 6. The average weights and tumor weight-inhibitions of mice before and after treatment (mean ± SD, n = 6).

| Groups      | Dose (mg/kg) | Tumor weights (g) | Tumor weight-inhibitions (%) |
|-------------|--------------|-------------------|-------------------------------|
| Control     | Solution     | 0.563 ± 0.187     | 3.00                          |
| Dox         | 2 mg/kg      | 0.244 ± 0.032**   | 58.44                         |
| TBESD       | 200 mg/kg    | 0.397 ± 0.049**   | 29.48                         |
| TBESD-ASDs  | 200 mg/kg    | 0.304 ± 0.042**   | 46.00                         |

**Indicates significant difference at p < 0.01.

Conclusions

In the present study, an amorphous TBESD solid dispersion was successfully prepared by the solvent evaporation method and subsequently characterized. The solubility and dissolution rate of the five ingredients were significantly increased following the formation of the solid dispersion with PVP K-30, a suitable hydrophilic polymer and drug excipient. The composition of the formulation and the preparative technique were subsequently optimized using an L9 (3^4) orthogonal experimental design. The five major ingredients exhibited chemical stability over the 90 days of monitoring. During this period, the TBESD-ASD was stored as capsules under accelerated conditions. Pharmacokinetic evaluation of the amorphous nature of the TBESD solid dispersion showed that the oral bioavailability of amentoflavone, robustaflavone, 2'-dihydro-3',3''-biapigenin, 3',3''-binaringenin, and delicaflavone were markedly improved and their RA reached 293%, 372%, 375%, 484%, and 278%, respectively. The significant increase in bioavailability was found to correspond with the increase in membrane permeability and bio-efficacy. In fact, the TBESD-ASD demonstrated greater antitumor effects than free TBESD, without systemic toxicity. These encouraging results suggest that ASD is an efficient drug delivery system for TBESD administration for cancer treatment, and could a promising strategy for improving oral bioavailability and reducing the dose required for therapeutic efficacy.

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Disclosure statement

No potential conflict of interest was reported by the authors.

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