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SCREENING FOR BIOLOGICAL ACTIVITIES OF THE TRADITIONAL CHINESE MEDICINE FANG FENG TONG SHEN SAN

Adelheid Brantner [*1], Muaaz Al-Ajlani [1], Yanyan Zhou [2], Haiyu Zhao [2] and Baolin Bian [2]

Institute of Pharmaceutical Sciences - Pharmacognosy [1], University of Graz, Universitaetsplatz 4/1, 8010 Graz, Austria.
Institute of Chinese Materia Medica [2], China Academy of Chinese Medical Sciences, No.16 Nanxiaojie, Dongzhimen Nei Ave., Dongcheng District, 100700 Beijing, PR China.

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Correspondence to Author:
Dr. Adelheid Brantner

Associate Professor,
Institute of Pharmaceutical Sciences, Pharmacognosy, University of Graz, Universitaetsplatz 4/I, 8010 Graz, Austria.

E-mail: adelheid.brantner@uni-graz.at

ABSTRACT: The ancient Chinese formulation Fang Feng Tong Shen San (FFTSS) consists of 17 components. The purpose of this study was to investigate the anti-inflammatory, antioxidant, anti-diabetic, anti-tyrosinase, anti-neuro-degenerative and antimicrobial effects of five commercial preparations of FFTSS and two laboratories constituted mixtures. *In-vitro* 2, 2-diphenyl-1-picrylhydrazyl (DPPH), lipid peroxidation (LPO), α-glycosidase, tyrosinase, acetylcholinesterase, butyrylcholinesterase, elastase inhibitory and antimicrobial assays were carried out in order to determine the active potential of FFTSS. All commercial preparations have shown antioxidant properties after applying DPPH and LPO assays of an average IC_{50} of 117.68µg/ml and 2.86µg/ml, respectively. In addition, all commercial preparations exhibited a moderate inhibition against tyrosinase of an average IC_{50} of 0.353mg/ml while the activity were higher in the formula without salts and excipients reaching an IC_{50} of 0.077mg/ml. The anti-neurodegenerative property of FFTSS was weak while the inhibitory activity of elastase was stronger. The formula was able to inhibit α-glucosidase in an average of 60.22%. FFTSS showed stronger effect against *Staphylococcus aureus* but less antimicrobial activity against Gram-negative bacteria. In summary FFTSS has shown efficient scavenging effects of superoxide and peroxide radicals as well as a strong anti-inflammatory and anti-diabetic potential. Therefore the formulation can be considered as a valuable source of a natural antioxidant and anti-inflammatory, anti-diabetic and anti-aging agent but also as a candidate in the search for modern pharmaceuticals.

INTRODUCTION: Traditional Chinese Medicine (TCM) is not only an ancient medicine but continues to be in use today. Indeed, it is still a major part of healthcare provision in China as it is provided in many state hospitals alongside standard medicines [1].

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According to Chinese clinical studies these medicines can greatly increase the effectiveness of modern drug treatments, reduce their side-effects, and sometimes replace them completely [2]. The scientific basis for the effectiveness of TCM formulations is not yet fully understood and therefore they are not used in modern western medicine [3]. Modernization of TCM is the key factor in the development of these reputable medicines. Chinese Herbal Medicine produced several hundred combinations for very effective and diverse treatments [4]. One of many formulas mentioned in the Chinese Pharmacopoeia is Fang Feng Tong Sheng San (FFTSS) [5].

FFTSS is a complex Chinese formula with 17 components including 14 different component herbs and 3 salts which are mixed to certain amounts. FFTSS dispels pathogenic wind (heat) from the body surface and purges away the dampness heat from the interior. FFTSS has many applications including asthma, obesity, vomiting and headache. However, for the formula to be used in modern medicine experimental evidence must be presented to prove its effectiveness. There is a lack of scientific studies that surrounds the TCM and their applications and interventions [6]. Five FFTSS preparations as well as two in-house preparations, one composed according to the Chinese Pharmacopeia [5] and another without salts was included in these investigations. For this purpose all FFTSS samples were tested with *in-vitro* bioassays for their 2, 2-diphenyl-1-picrylhydrazyl (DPPH), tyrosinase, lipid peroxidase (LPO), elastase, α–glycosidase, acetylcholinesterase, and butyrylcholinesterase inhibition as well as for their inhibitory effect against different strains of bacteria and yeasts. Variations could derive from the chemical constituents of the 14 component herbs. The yield of the ingredients can be influenced by various parameters like the plant origin, cultivation and collection conditions and processing procedures [7]. In the present study the anti-oxidative, anti-inflammatory, anti-diabetic, anti-neuro degenerative, and anti-aging potentials along with the antimicrobial activity of FFTSS have been examined to provide accurate, solid evidence of their biological activities.

MATERIALS AND METHODS:

FFTSS Formulation and Component Herbs: FFTSS is a complex Chinese formula with 17 components including 14 different herbal plants and 3 salts that are mixed in a certain ratio **(Table 1)**. Radix Saposhnikoviae, Herba Ephedrae, Radix et Rhizoma Rhei, Herba Schizonepetae tenuifoliae, Herba Menthae haplocalycis, Fructus Gardeniae jasminoidis, Fructus Forsythiae suspensae, Radix Scutellariae baicalensis, Radix Platycodi grandiflori, Radix Ligustici striati, Radix Angelicae sinensis, Radix Albus paeoniae lactiflorae, Radix Atractylodis macrocephalae, Radix Glycyrrhizae uralensis, Talcum, Gypsum fibrosum and Natrii sulfas were purchased from "Tian Gang Pu Ren Da Yao Fang Store" in Beijing, China (2016) and authenticated by Dr Haiyu Zhao, China Academy of Chinese Medical Sciences, Beijing, China **(Table 1)**.

The plant material and the in-house preparations FFTSS6 and FFTSS7 were deposited at the Institute of Pharmaceutical Sciences. Pharmacognosy, University of Graz, Austria. Five FFTSS preparations from different Chinese manufacturers as well as two in-house preparations were included in these investigations. Sample FFTSS6 was prepared by mixing the dried plant material in ratio recommended by the Chinese Pharmacopoeia [5] but without the three salts. Sample FFTSS7 contained the complete formula including the three salts **(Table 2)**.

TABLE 1: COMPOSITION OF FANG FENG TONG SHEN SAN AND THE CHINESE INDICATIONS OF THE INDIVIDUAL COMPONENTS

Plant Part	Plant	Pin Yin	Ratio	Actions
Rd. Saposhnikoviae	*Ledebouriellae divaricatae* Turcz.	Fang Feng	1	Releases the exterior, expels external wind
Hb Ephedrae	*Ephedra sinica* Stapf.	Ma Huang	1	Releases the exterior moves lung Qi, stops wheezing and promotes urination
Wine-treated Rd. et Rhiz. Rhei	*Rheum palmatum* L.	Jiu Da Huang	1	Drains heat, purges accumulations and reduces fire toxicity
Hb. Schizonepetae tenuifoliae	*Schizonepatae tenuifoliae* Briq.	Jing Jie	1	Releases the exterior, expels wind, moves Qi, vents rashes and relieves itching
Hb. Menthae haplocalycis	*Menthae haplocalycis* Briq.	Bo He	1	Disperses wind-heat, cools and clears the head, benefits the throat and vents rashes
Fr. Gardeniae jasminoidis	*Gardeniae jasminoidis* J. Ellis	Zhi Zi	1	Clears heat, reduces fire and eliminates irritability
Fr. Forsythiae suspensae	*Forsythiae suspensae* L.	Lian Qiao	1	Clears heat, relieves toxicity and expels external wind-heat
Rd. Scutellariae baicalensis	*Scutellaria baicalensis* Georgi	Huang Qin	2	Clears lung heat and drains fire

Rd. Platycodi grandiflori	*Platycodi grandiflorae* Jacq.	Jie Geng	2	Opens lungs, spreads lung Qi, expels phlegm, expels pus, and benefits the throat
Rd. Ligustici striati	*Ligusticum striatum* DC.	Chuan Xiong	1	Activates blood, regulates Qi, expels wind-cold, alleviates pain and treats headache
Rd. Angelicae sinensis	*Angelicae sinensis* (Oliv.) Dies.	Dang Gui	1	Moistens intestines, unblocks bowels, reduces swelling, expels pus, and alleviates pain
Rd. Albus paeoniae lactiflorae	*Paeoniae lactiflorae* Pall.	Bai Shao	1	Nourishes blood and alleviates pain
Rd. Atractylodis macrocephalae	*Atractylodis macrocephalae* Koidz.	Bai Zhu	1	Tonifies the spleen and tonifies Qi to protect the spleen
Rd. Glycyrrhizae uralensis	*Glycyrrhizae uralensis* Fisch.	Gan Cao	4	Tonifies the spleen, tonifies Qi, moistens the lungs, resolves phlegm, stops cough, clears heat, relieves fire toxicity and benefits the throat to improve swallowing
Talcum	-	Hua Shi	6	Promotes urination and drains heat
Gypsum fibrosum	-	Shi Gao	2	Clears heat in the Qi stage, drains fire, clears excess heat from the lungs, clears blazing stomach fire, heals sores and wounds, nourishes body fluids and stops thirst
Natrii sulfas (Mirabilitium)	-	Mang Xiao	1	Purges accumulations, eliminates stagnation, and clears heat

TABLE 2: SOURCES AND CONSTITUENTS OF FANG FENG TONG SHEN SAN AND IN-HOUSE PREPARATIONS

Code	Manufacturer	Formulation	Patch No.
FFTSS1	China Beijing Tong Ren Tang Technologies Co., Ltd	pills not coated [1)]	14081608
FFTSS2	Jiangsu Pingguang Xinyi (Jiaozuo) Chinese Medicine Co., Ltd	pills not coated [1)]	150401
FFTSS3	Changchun Overseas Pharmaceutical Group Ltd	pills coated [1)]	20131101
FFTSS4	Shangquin City, Kinmen and Matsu Pharmaceutical Co., Ltd	pills coated [1)]	14050321
FFTSS5	Yantai Bohai Pharmaceutical Group Co., Ltd	granules [1)]	150207
FFTSS6	14 herbal components	dry powder	In-house
FFTSS7	14 herbal components + 3 salts	dry powder	In-house

[1)] Pills/granules composed of the aqueous extract of FFTSS

Chemicals: All chemicals were of analytical grade. Natrium di-hydrogenphosphate dihydrate, di-natrium hydrogen phosphate dihydrate, *n*-hexane, dichloro-methane (DCM), methanol, *n*-butanol, dimethyl sulfoxide (DMSO), rutin trihydrate, and quercetin dihydrate were purchased from Roth (Karlsruhe, Germany). Triton X-100, 3,4-dihydroxy-l-phenylalanine (L-DOPA, Sigma, Art, No.D9628), L(+)-ascorbic acid (Roth, Art, No.3525.1), tyrosinase from mushrooms (Sigma, Art, No.T3824), 2,2-diphenyl-1-picrylhydrazyl radical (DPPH), butylated hydroxytoluene (BHT), 2-thiobarbituric acid (TBA), phosphate-buffered saline pH 7.4 was purchased from Sigma-Aldrich (Vienna, Austria). Methanol (MeOH) was purchased from VWR (Darmstadt, Germany), sodium hydroxide (NaOH) and iron III chloride ($FeCl_3$) from Merck (Darmstadt, Germany), trichloroacetic acid (TCA) from Fluka (Vienna, Austria), Tris HCl (Roth, Art.No.9090.1), SucAla3-PNA (Sigma, Art.No.S-4760), Elastase from hog pancreas (Fluka, Art.No.45125), Trizma Pre-set crystals (Sigma, Art.No.T8443), 5,5'-Dithiobis (2-nitro-benzoic acid) (Sigma, Art.No.D8130), Acetylthiocholine iodide (ATCI, Sigma, Art. No. A5751), AChE from Electophorus electricus electric eel Type VI-S (AchE, Sigma, Art.No.C3389), S-Butyrylthiocholine iodide (BTCI, Fluka, Art.No.20820), BuChE from equine serum (BuChE, Sigma, Art.No.C7512), Physostigmine (eserine) (Sigma, Art.No.E8375) and 96 well/flat bottom microtiter plates from Sterilin (Cambridge, UK).

Microorganisms: *Candida albicans* ATCC 10231, *Candida parapsilosis* ATCC 90018, *Candida tropicalis* ATCC 13803; *Proteus mirabilis* ATCC 14153, *Klebsiella oxytoca* ATCC 8724, *Staphylococcus epidermidis* ATCC 12228, *Staphylococcus aureus* subsp. *aureus* ATCC 25923, *Pseudomonas aeruginosa* ATCC 27853, *Bacillus subtilis* subsp. *subtilis* ATCC 6051, and *Escherichia coli* ATCC 25922 were used as test organisms.

Antibiotics and Media: The reference antibiotics kanamycin sulphate, tetracycline HCl, gentamycin sulphate, penicillin G, ampicillin, and fluconazole were purchased from Serva, Heidelberg, Germany. Iso-Sensitest agar (for bacteria) and Sabouraud dextrose agar (for yeasts) were obtained from Oxoid, Basingstoke, UK.

Preparation of Extracts: The four preparations (FFTSS1-4) were pills. FFTSS1 and FFTSS2 were non-coated pills while FFTSS3 and FFTSS4 were coated pills with sugar. FFTSS5 were granules. FFTSS6 and FFTSS7 were composed following the monograph of the Chinese Pharmacopoeia [5] **(Table 2)**. FFTSS6 did not include salts. 10g of each sample were suspended in 30ml methanol. After ultrasonication for 30 min the samples were filtered. The filtrates were dried in vacuum. 1mg of each powdered extract was dissolved in 1ml DMSO and used as stock solution.

DPPH Radical Scavenging Assay: The DPPH (2, 2-diphenyl-1-picrylhydrazyl) radical scavenging activity of the formulations, herbal drugs and pure compounds was determined according to a modified method of Mensor *et al.*, [8]. Defined amounts of each sample were dissolved in MeOH. 75µl of samples/pure compounds were incubated with 75µl of the DPPH solution (0.3mM) in the dark for 30 min at 27 °C. The blank consisting of 75µl sample and 75µl solvent was treated in the same way. The kinetic decrease in DPPH absorbance was measured at 517 nm with a Wallac 1420 microplate reader. The scavenging capacity of the samples was compared with that of the solvents. Rutin was used as positive control.

Tyrosinase Inhibition Assay: Tyrosinase inhibition activity was determined using a modified dopachrome method with L-DOPA as substrate [9]. All samples were dissolved in DMSO containing 5% Triton. 20µl of the extracts, 40µl of tyrosinase (100U/ml) and 100µl buffer (88 ml $Na_2HPO_4.2H_2O$ with 97ml of $NaH_2PO_4.2H_2O$ at pH 6.8) were mixed. Each sample was accompanied by a blank containing all the components except tyrosinase. The results were compared with a control consisting of 20µl DMSO, 40µl tyrosinase (100U/ml) and buffer solution. Sample, blank, and control were incubated at 29 °C for 10 min. 40µl of L-DOPA (12.5mM) were added to each well as substrate. The amount of dopachrome was measured immediately and every 4 min at 490nm using a microplate reader. Ascorbic acid was used as positive control.

Non-Enzymatic Lipid Peroxidation Inhibition Assay: The seven samples were investigated as possible inhibitors of membrane lipid peroxidation using a previously described method of Houghton *et al.*, [10] with some minor modifications. Phospholipid liposomes were prepared by mixing bovine brain extract Type VII with phosphate-buffered saline (5mg/ml, pH 7.4) followed by sonication at 0-4 °C until opalescent. The lipid peroxidation was stimulated by mixing 500µl bovine brain extract, 300µl of the phosphate-buffered saline including the sample which was first dissolved in DMSO and then diluted with buffer and 100µl of $FeCl_3$. Peroxidation was initiated by adding 100µl1mM ascorbic acid. After incubation for 1h at 37 °C the extent of the liposome lipid peroxidation was assessed using thiobarbituric acid [11]. 100µl of the reaction mixture were added to 100µl of 1% thiobarbituric acid, 100µl of 2.8% trichloroacetic acid and 10µl of 2% butylhydroxytoluene. After heating for 20min at 100 °C in a water bath, the mixture was cooled to 25 °C. 250µl of *n*-BuOH were added for extraction of the chromogen and the tubes were centrifuged for 5 min at 3500 rpm. The organic layer was transferred to microtiter plates and the absorbance of the pink pigment was measured at 532nm. A mixture without bovine brain extract was used as blank, and a mixture without sample was used as control. Quercetin dihydrate was applied as positive control.

Elastase Inhibition Assay: The elastase activity was examined using *N*-succinyl-Ala-Ala-Ala-*p*-nitroanilide as substrate. The reaction was carried out in 200 mmol L^{-1} Tris-HCl buffer (pH 8.0) containing 0.2 mmol L^{-1} *N*-Suc-(Ala) 3-nitroanilide and 0.104U ml^{-1} elastase [12]. FFTSS extracts were added to the reaction mixture and the elastase inhibition was assessed at 25 °C. The reaction mixture was pre-incubated for 10 min before addition of the substrate. The change in absorbance by the release of p-nitroaniline was measured at 410nm in a microplate reader.

α-glycosidase Inhibitory Assay: The enzyme, inhibitor and substrate solutions were prepared using the same phosphate buffer (50 mM; pH 6.8) [13]. A reaction mixture containing 50µlof the buffer, 10µl of α-glucosidase (1U/ml) from *Saccharomyces cerevisiae* and 20µl of each sample were transferred to 96-well plates. 20µl of 1mM 4-nitrophenyl-α D-glucopyranoside was added to the mixture as substrate. Incubation was carried out at 37 °C for 30 min. Acarbose was used as positive control and water as negative control. The yellow p-nitrophenol was measured spectrophotometrically at 405nm, 37 °C, every 30 sec for 5 min. Each experiment was performed in triplicate along with appropriate blanks.

Acetylcholinesterase Inhibition Assay: The enzymatic activity was measured using a modified method described by Ingkaninan *et al.,*[14] 65µl of Trisma buffer (pH 8), 20µl of the extracts dissolved in DMSO in different concentrations and 5µl of an enzyme solution in a concentration of 0.28U/ml were incubated during 30 min at 4 °C. Subsequently, 15µlof a solution of acetylthiocholine iodide (ATCI) (0.023mg/ml) and 95µl of 3mM DTNB were added. The final mixture was incubated for another 20min at room temperature. The absorbance of the mixture was measured photometrically at 405nm. A control was prepared using solvent instead of the extract.

Butyrylcholinesterase Inhibition Assay: The inhibitory activity was measured by a spectrophotometric method [15]. The reaction mixture contained 190µl Trizma buffer (100mM) (pH 8.0), 20µl of the sample and 40µl of butyrylcholinesterase solution (0.035U/ml) were mixed and incubated for 30min (4 °C). The reaction was initiated by the addition of 20µl DTNB (10mM) and 20µl butyrylthiocholine iodide (10mM) as substrate. The mixture was incubated for 20min at 37 °C. The hydrolysis of butyrylthiocholine was monitored by the formation of the yellow 5-thio-2-nitrobenzoate anion as a result of the reaction of DTNB with thiocholine which was released by the enzymatic hydrolysis of butyrylthiocholine. The measurement was performed at a wavelength of 412nm (15 min).

Calculation: All test enzymatic assays were carried out in triplicate and the data presented as mean ± SD. The percentage inhibition was evaluated by the following equation:

$$Inhibition(\%) = 100 \cdot \frac{(\Delta A_{control} - \Delta A_{sample})}{\Delta A_{control}}$$

Antimicrobial activity: All samples of FFTSS were tested against a number of microorganisms at concentrations of 10µg/mL in DMSO at the recommended growth conditions from the European Pharmacopoea [16]. The agar diffusion method was employed for monitoring the antimicrobial activity. The samples were transferred to sterile discs (diameter 6mm) which were placed on the agar seeded with microorganisms. The inhibition zone diameters were measured for evaluation. The test was performed in triplicate.

RESULTS:

TABLE 3: INHIBITORY EFFECTS OF FFTSS FORMULATIONS OF THE DPPH, TYROSINASE, LPO, ELASTASE, ACETYLCHOLINESTERASE, BUTYRYLCHOLINESTERASE AND A-GLYCOSIDASE INHIBITORY ASSAYS (IC_{50} µg/ml)

Formulations / references	DPPH	Tyrosinase	LPO	Elastase	Acetylcholin-esterase	Butyrylcholin-esterase	α-Glycosidase (%)
FFTSS1	109.57±3.25	186±6.46	3.31±0.86	0.26±1.46	538.00 ±2.18	388.09±3.22	59.04±1.50
FFTSS2	143.86±4.65	427±3.43	2.16±1.65	1.29±1.82	112.52±3.92	208.29±3.02	48.55±1.60
FFTSS3	86.89±5.29	509±4.76	3.04±0.38	0.41±3.46	427.92±2.03	301.18±2.38	58.33±2.00
FFTSS4	161.63±2.95	111±7.66	5.26±1.06	0.45±1.90	361.96±3.42	362.86±3.53	56.95±3.20
FFTSS5	86.46±5.64	534±1.27	0.26±2.48	0.33±2.11	115.76±1.23	501.30±2.18	78.24±9.80
FFTSS6	140.75±3.24	77±1.06	1.70±0.97	0.07±1.49	12.93±4.98	136.12±4.94	87.76±6.30
FFTSS7	127.23±2.72	202±2.25	2.27±0.79	0.35±2.59	230.14±3.23	269.16±2.65	87.68±7.10
Ascorbic acid		36.73±0.68	-	-	-	-	-
Rutin	11.85±0.86	-	-	-	-	-	-
Quercetin.dihydrate	-	-	1.70±1.86	0.48±1.32	-	-	-
Eserine	-	-	-	-	0.46±3.48	0.58±2.92	-
Acarbose	-	-	-	-	-	-	92.63±0.90

Values are the mean ± SD (n = 3); FFTSS Fang Feng Tong Shen San preparations; - not determined

DPPH Radical Scavenging Activity: Table 3 reveals that the commercial Fang Feng Tong Shen San preparations showed antioxidant properties to a more or less similar extent. The activity was also similar for the in-house preparations FFTSS6 and FFTSS7.

Tyrosinase Inhibitory Activity: The results of the tyrosinase inhibition assay carried out on the methanolic extracts of the formulations are shown in **Table 3**. The preparation FFTSS6 without salts demonstrates the strongest activity (77±1.06 µg/ml). All samples inhibited the oxidation of L-DOPA catalyzed by mushroom tyrosinase in a dose-dependent manner. The inhibition of tyrosinase is one of the weakest biological activities of Fang Feng Tong Shen San.

Lipid Peroxidation Inhibitory Activity: The results obtained from the seven preparations showed some dissimilarity by comparing the values of the LPO inhibition (**Table 3**). The granules of FFTSS5 showed a seven fold stronger inhibition than the standard quercetin. It is advantageous that granules possess less excipients and no additional coating material. In the same way FFTSS6 is more active because the formulation contains more plant material due to the absence of the salts. The inhibition rate is equal to that of the reference quercetin. The formulated preparations FFTSS1, FFTSS2, FFTSS3, and FFTSS4 exhibited a weaker activity on the LPO inhibition. In conclusion the formula shows pronounced inhibitory effects over LPO strongly depending on the formulation. These results may have a positive impact to the clinical application of the formulation.

Elastase Inhibitory Activity: The inhibitory values for elastase obtained from all FFTSS preparations were comparable to the reference quercetin **(Table 3)**. In the in-house preparation FFTSS6 without salts the elastase inhibitory effect is highest with an IC_{50} of 0.07±1.49 µg/ml exceeding the reference quercetin with an IC_{50} of 0.48±1.32 µg/ml nearly seven times. The inhibitory effect of the enzyme is lower when the salts are added to the formulation as shown with the sample FFTSS7 (IC_{50} 0.35±2.59 µg/ml).

Butyrylcholinesterase and Acetylcholinesterase Inhibition Assay: The anti-neurodegenerative assays showed a very low score among all investigated FFTSS formulations. The extent of inhibitions was moderate but similar for both enzymes. The best result among the preparations was achieved by the in-house preparation FFTSS6 containing only component herbs. All other preparations showed similar weak activity compared to the reference eserine (**Table 3**).

α-glycosidase Inhibitory Assay: α-glycosidase is a key enzyme in carbohydrate digestion. The samples were compared to acarbose which acts as an inhibitor of the α-glycosidase. It can be used in the treatment of Diabetes Type II. So the ability of FFTSS is studied to inhibit this enzyme responsible for the breakdown of complex sugars into smaller molecules to make them available for the intestinal absorption. The results are shown in **Table 3**. The formula considerably inhibits α-glucosidase with the highest inhibition achieved by the commercial preparation FFTSS5 (78.24±9.80%) in comparison to acarbose (92.63±0.90%) showing another clear advantage of the granular formulation. The other commercial preparations showed α-glycosidase inhibitory effects between 48.55±1.60% and 59.04±1.50%.

TABLE 4: ANTIMICROBIAL ACTIVITY OF SEVEN FFTSS PREPARATIONS

Microorganism	Antibiotic	FFTSS1	FFTSS2	FFTSS3	FFTSS4	FFTSS5	FFTSS6	FFTSS7
Candida albicans ATCC 10231	24 [F]	<7	8	11	8	10	11	10
Candida tropicalis ATCC 13803	36 [F]	<7	<7	<7	<7	<7	10	<7
Staphylococcus epidermidis ATCC 12228	13 [T]	9	9	9	9	9	13	9
Staphylococcus aureus subsp. *aureus* ATCC 25923	17 [K]	12	11	10	9	12	13	12
Bacillus subtilis subsp. *subtilis* ATCC 6051	13 [K]	9	9	8	8	8	10	8
Escherichia coli ATCC 25922	14 [K]	8	<7	<7	<7	9	8	9

*Antibiotics: [K] Kanamycin, [T] Tetracycline, [F] Fluconazole

Inhibition zone diameters in mm; mean values N=3; as the disc diameter was 6mm inhibition zones <7mm were not evaluated.

The two in-house mixtures FFTSS6 and FFTSS7 demonstrated similar results showing a strong inhibitory effect of 87.76±6.30% and 87.68±7.10%, resp. similar to that of acarbose (92.63±0.90%).

Antimicrobial Activity: The antibacterial activity of the FFTSS samples was more prominent against Gram-positive bacteria especially *Staphylococcus* species. The laboratory formulations FFTSS6 and FFTSS7 inhibited a larger number of microorganisms in comparison to the commercial preparations. The reason therefore might be that they contain no additives from the manufacturing process. The activity can be also lost as a consequence of the extraction process for the commercial products. FFTSS6 and FFTSS7 were extracted freshly without any manufacturing steps that could have undermined their effectiveness. All FFTSS samples showed an inhibition of *Staphylococcus aureus* subsp. *aureus* with the exception of FFTSS4 which was less potent. None of the FFTSS samples inhibited the growth of *Candida parapsilosis, Proteus mirabilis, Klebsiella oxytoca* and *Pseudomonas aeruginosa*.

DISCUSSION: This study was set up to investigate the anti-oxidative, anti-inflammatory, anti-diabetic, anti-neurodegenerative, anti-aging and antimicrobial activities *in vitro* of the complex TCM formulation FFTSS. Therefore the results of five commercial samples from different Chinese manufacturers were compared with two in-house prepared formulations to avoid the influences of excipients or procedures of manufacturing. One formulation was containing the 14 component herbs and the other additionally three salts. Oxidative stress and inflammation processes may contribute too many complex chronic diseases like neurodegenerative disorders in which the treatment with TCM can be useful. These symptoms often come together and are treated separately in modern medicine. The FFTSS indications are wide and could be effective in dealing with such a medical situation in general. Flavonoids as ingredients of this formula possess a wide range of bioactive effects including strong antioxidant activity [17]. These antioxidant agents can scavenge free radicals that protect the organism against damage caused by free oxygen species [18, 19]. The free radicals are mainly reactive oxygen (ROS) and reactive nitrogen (RNS) species. They are products of normal cellular metabolism and cause various damages to the cellular structures when occurring in high concentrations [20, 21]. If these cellular structures undergo the process of oxidation, they may assist in the development of chronic diseases such as rheumatism, cancer, cardiovascular and neurodegenerative diseases [22, 23]. The antioxidant effect on the other hand stabilizes these free radicals and marks them as controllable. The antioxidant activity of all FFTSS formulations was not as strong as expected referring to the polyphenolic content of the component herbs.

Antioxidant and anti-tyrosinase effects play an important role in melanogenesis which is activated by oxidation-related processes such as UV radiation. It requires the activity of the enzyme tyrosinase as well as reactive species like reactive oxygen and nitrogen species causing oxidative stress to cells, especially of skin cells, which results in pigmentation and aging [24]. Tyrosinase expression and activation play an essential role in melanin production. Although melanin protects the skin against UV injury under normal physiological conditions, abnormal pigmentation such as freckles and age spots often causes serious skin problems and esthetic issues [25]. A few known natural tyrosinase inhibitors of melanin synthesis including adenosine, arbutin, niacinamide, and kojic acid are currently used as cosmetic and medicinal ingredients in preparations applied for the treatment of hyperpigmentation. Most skin-whitening ingredients often have carcinogenic potentials as well as weak efficacies and therefore there is a clear need for the development of safer and more efficient ingredients in this regard. FFTSS preparations could potentially be an alternative medicine for the treatment of skin aging.

Additionally to the anti-aging property the inhibition of elastase will allow slow skin aging but also treatment of many skin disorders. The *in vitro* results demonstrate that FFTSS is inhibiting the enzyme up to seven times stronger compared to the reference quercetin. Lipid peroxidation is a well-defined mechanism of cellular damage that occurs *in vivo* during aging and in certain disease states. Lipid peroxides are unstable markers of oxidative stress. The extracts of all investigated FFTSS formulations showed a strong lipid peroxidation inhibitory activity in the applied *in vitro* assay.

The α-glycosidase is an enzyme that breaks down complex sugars and makes them available for absorption into the blood stream. By the inhibition of α-glycosidase in the intestine, the rate of hydrolytic cleavage of oligosaccharide is decreased and the process of carbohydrate digestion spreads to the lower part of the small intestine. The inhibition produced by FFTSS to such enzyme will have a dramatic effect in reducing the sugar level in the body and thus the anti-diabetic property and anti-obesity can be correlated. The formulation showed a strong anti-diabetic effect which was demonstrated mainly with the in-house preparations in comparison to acarbose. This type of treatment could be a unique class of anti-diabetic medicine against diabetes Type II and a specific target for chronic predictable cases in the aged population. By competitively inhibiting glycosidase activity, these inhibitors help to prevent the fast breakdown of sugars and thereby may control the blood sugar level. This can support the use of FFTSS for weight reduction.

Inhibition of butyrylcholinesterase and acetylcholinesterase, the key enzyme in the breakdown of acetylcholine, is considered as a promising strategy for the treatment of neurological disorders such as Alzheimer's disease, senile dementia, ataxia and myasthenia gravis by potentiating and affecting the cholinergic transmission process. The FFTSS formulas hold some activities against both enzymes but the extent of inhibition was not substantial. The activity of all FFTSS preparations against some Gram-positive bacterial strains justifies the application of this formulation in the treatment of acne in Chinese therapy. *Staphylococcus aureus* is involved in this disease and a bactericidal effect of FFTSS could be observed against this bacterial strain.

Chinese herbal medicines are complex systems. The pharmacological activities mostly cannot be related to a single compound because the combination of more than one active ingredient may contribute to the therapeutic effect. Therefore it is necessary and sometimes more useful to evaluate the activities of the TCM formulations as such rather than focusing in a single active ingredient. The main groups of compounds present in these formulations are terpenes, flavonoids, saponins, alkaloids and volatile oils. As an example the test results of baicalin and baicalein should be mentioned. Baicalin and baicalein are main ingredients of Radix Scutellariae which is one of the most prominent component herbs of FFTSS. The two compounds were tested for their anti-oxidative and anti-inflammatory activities in a previous study [26]. The FFTSS samples tested with the non-enzymatic lipid peroxidation inhibition assay resulted in an average IC_{50} of 2.86µg/ml in comparison to the IC_{50} of the reference quercetin which is 1.70µg/ml. The pure compounds baicalin and baicalein showed a weaker effect with an IC_{50} of 5.37µg/ml and 3.49µg/ml, respectively. The fact that fourteen plants are involved in this formula may produce a complex synergism or addition of the activity.

In clinical practice, the original prescription of FFTSS can be modified to focus on certain effects. Jia Wei Fang Feng Tong Sheng Wan, for example, is a modification intended to reduce weight and consequently obesity. These particular effects are aligned perfectly with the anti-diabetic effect of FFTSS as obesity often is a condition of diabetic patients. The later property is basically due to the inhibition of α-glucosidase that will reduce the sugar absorption and ultimately lead to weight reduction. The broad clinical applications of FFTSS require a systematic study to understand such clinical indications. The panel of biological activities tested in this paper will help to determine the effectiveness, role and possible mechanisms of action of FFTSS in Traditional Chinese Medicine. These data of FFTSS are of great interest and will guarantee the propagation of Chinese Medicine to the world.

CONCLUSION: From the present study it can be concluded that both commercial and laboratory FFTSS preparations have similar effects in the applied *in-vitro* bioassays though slight preferences towards the in-house preparations. The inhibitory effects of the granules were more pronounced compared to the five commercial preparations. Finally, the FFTSS formulations embrace many activities and therefore they represent an ideal combination for the holistic approach of the treatment of patients.

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