

Glycyrrhiza Glabra and *Platycodon Grandiflorus* are Alternative Treatment Options for Vocal Fold Nodules

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Research

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Abstract

Background: Vocal fold nodules (VFN) are one of the main causes of hoarseness. In this study, we try to figure out an alternative treatment from our clinical experience summary.

Methods: We retrospectively reviewed VFN patients who received traditional Chinese medicine (TCM) treatments from July 2018 to August 2020 and traced their Chinese Voice Handicap Index-10 (VHI-C10) and multidimensional voice program (MDVP) analysis results. Also, we induce inflammatory response of porcine vocal fold epithelial (PVFE) cells with 50 ng/mL TNF-alpha. The inflamed PVFE cells were separately cultured in the aqueous extract of *Glycyrrhiza glabra* and *Platycodon grandiflorus*.

Results: For these patients ($n = 22$), the average VHI-C10 score decreased from 17.6 to 6.6 ($p < 0.001$). MDVP analysis revealed improvements in jitter, shimmer, noise–harmonic ratio, and GRBAS scoring system. Analysis of prescription patterns revealed that the most commonly used herbs were Licorice and *Platycodon grandiflorus*. In the MTT assay of PVFE cells, no adverse effects of our extracts were observed at doses of 1–200 ug/mL. Western blot analysis revealed downregulation of p65 and mitogen-activated protein kinase pathway proteins.

Conclusions: The results from both the clinical and *in vitro* aspects of this study revealed that the herbs *G. glabra* and *P. grandiflorus* may offer beneficial outcomes as alternative treatments for VFNs.

Introduction

Vocal fold nodules (VFNs) as benign vocal fold lesions, is quite a challenge for otolaryngologists. Hoarse and raspy voices are distinguished symptoms for VFNs. The definition of VFNs are mucosal lesions occurring on both sides of the vocal fold on the border of the anterior and middle third of the vocal fold (1, 2). Such lesions are often found on the superficial layer of the lamina propria (3). Long term exposure to irritants may cause chronic laryngeal inflammation, which may further lead to the formation of VFNs (4).

Medical treatments, voice therapy and excision through microlaryngeal surgery are considered as VFN treatment. Physiological, medical, and psychological factors should be taken into account in the course of diagnosis and treatment. (5, 6).

Voice therapy, which can alter voice production patterns to minimize phonotrauma, is the first-line treatment for VFNs because it typically resolves voice problems and prevents recurrence in most patients. Voice therapy typically involves education on vocal fold mechanics and etiological factors, as well as specific modifications to behaviors that exacerbate inappropriate voice production (5). However, because compliance varies among patients, surgery is often necessary for VFN treatment. However, serious complications associated with surgery may occur, including damage to the vocal folds (7). Intracordal steroid injection can be beneficial for managing benign VFNs. However, the technique requires a high level of skill and therefore a well-trained laryngologist. Nevertheless, VFNs may persist after injection.

Therefore, there are increasing patients turning to complementary medicine other than western medicine. (8) Because some of them may fear surgery or be concerned about using steroids, patients have recently shown increased preference for treatments outside the mainstream medical practice (9).

As for physiotherapy, such as massage, spinal manipulative therapy and acupuncture, there are several trials revealing that these treatments might be effective for voice disorders. (10) In an small-scaled acupuncture study, anti-inflammatory significantly increased was found in the experiment group. This may be the mechanism why complementary medicine is effective to the voice disorders. (11)

When it comes to herbs, though there are scanty reports showing that herbs formula have significant effect on vocal nodule and vocal polyp patients.(12, 13) However, the methodological quality remained unsatisfactory. Also, the exact contents and the dosage were unclear in the above researches. Therefore, it is hard to ascertain the exact effect of each herb, not to mention the *in vitro* evidence of the herbs effect.

For *in vitro* part, some herbal aqueous and ethanol extracts such as clove and ginger exhibit anti-inflammatory ability in human tonsil epithelial cells. (14) However, cell types in the upper airway vary. The vocal fold epithelial cells are stratified squamous. (15) Since it is difficult to obtain healthy human vocal fold epithelial cells without risking vocal function, the fresh porcine vocal fold tissue is readily and easily available. (16) Moreover, compared to other animals, the morphology of porcine vocal fold epithelium is the most similar to that of humans. (17)

So far, few researches study the effects of herbs on both vocal fold nodule patients and inflammatory vocal fold cells. In our study, we retrospectively evaluated the clinical effects according to the clinical results of our TCM herbal treatment. Also, we further investigated the effects of these herbs in an *in vitro* study.

Materials And Methods

Subjects

The medical records of 22 patients who had received diagnoses of VFNs are based on videostroboscopy or final pathology from July 2018 to August 2020. Other benign vocal fold lesions were excluded from the present study, such as vocal polyps, vocal cysts, and Reinke's edema. The patients were examined with laryngeal videostroboscopy using a Kay Elemetrics Stroboscopy Unit (Model, Lincoln Park, NJ, USA) at their initial visit and were followed up 3 months after TCM treatment.

Speech pathologist skilled in voice training and an otolaryngologist evaluated the recorded data. Both of them were blinded to the TCM treatment protocols. The present study was approved by the Institutional Review Board of Chiayi Chang Gung Memorial Hospital (No.201901034B0). All participants gave written informed consent prior to the beginning of the study.

Objective and Subjective Voice Analysis

The acoustic parameters recorded automatically were as follows: average fundamental frequency (F0, in Hertz), jitter, shimmer, and noise-to-harmonic ratio (NHR). Jitter is defined as the cycle-to-cycle variation in fundamental frequency (18, 19), and shimmer is expressed in decibels, representing the variability in the peak-to-peak amplitude. NHR is used for evaluation of dysphonic voice by measuring the amount of additive noise in the voice signal (20). The acoustic parameters were measured by the Computerized Speech Laboratory (core model CSL 4500, KayPentax, Lincoln Park, NJ, USA). The duration parameter measured in this study was the maximum phonation time (MPT). A speaker's MPT is measured by a stopwatch, as their best attempt to sustain the vowel /a/ sound at a comfortable intensity. (21). Twenty-two patients completed a pre- and post-test Chinese Voice Handicap Index–10 questionnaire (VHI-C10) (22, 23). Pre- and post-TCM perceptual evaluation was performed by using the GRBAS scoring system (G = grade, R = roughness, B = breathiness, A = asthenia, and S = strain; 0 = normal, 1 = mild, 2 = moderate, and 3 = severe). If the difference between the two GRBAS scores exceeded two points, a reassessment is required. The voice parameters mentioned above were measured by a speech pathologist and an otolaryngologist in a double-blinded manner.

Aqueous Extracts of TCM Herb *Glycyrrhiza glabra* and *Platycodon grandiflorus*

The raw herbs used for the aqueous extracts (AE) were obtained from Chiayi Chang Gung Memorial Hospital (Taiwan), 20 g *G. glabra* from Batch No. 42050309 and 20 g *P. grandiflorus* from Batch No. N15529. The raw herb materials were separately macerated in 1000 mL reverse osmosis water and then boiled for 10 minutes. These filtrates were combined and concentrated under reduced pressure at 40°C by using a vacuum rotary evaporator to obtain crude AEs. Approximately 4 g of the resulting *G. glabra* AE (20% yield) and 11 g of the resulting *P. grandiflorus* AE (55% yield) were stored at – 20°C before use.

Primary Culture PVFE Cells

The porcine vocal fold epithelial (PVFE) cells for the primary culture were purchased from GeneDirex, Inc. The PVFE cells were isolated and cultured as reported. (16) Epithelial cells were derived from porcine vocal folds, and then further expanded in culture. Epithelial cells identification and characterization were done by immunostaining with pan-Cytokeratin antibodies.

MTT Assay

PVFE cells were treated with various concentrations of *G. glabra* and *P. grandiflorus*; subsequently, the percentages of metabolically active cells were determined based on the mitochondrial conversion of MTT into formazine. In brief, after cells were treated with *G. glabra* and *P. grandiflorus* for various incubation times, culture media were replaced with DMEM/F-12 (1:3 ratio) containing 0.02% MTT (Sigma-Aldrich) and incubated for 4 h; subsequently, the medium was replaced with 200 µL of dimethyl sulfoxide per well. The results were assessed in a 96-well format plate reader by measuring the absorbance at a wavelength of 595 nm on a Victor Nivo Multimode microplate reader (PerkinElmer, Akron, OH, USA).

Inflammatory Response Induction and Culture in TCM Aqueous Extract

The PVFE cells were seeded at a density of 35×10^3 cells/well in PLL-coated 24-well plates and incubated overnight at 35°C for 20 h. PVFE cells were then exposed to the inflammatory cytokine tumor necrosis factor alpha (TNF- α) 50 ng/mL for 24 h incubation at 35°C to trigger inflammation. In addition, the corticosteroid dexamethasone was included as a positive control anti-inflammatory drug.

Western Blot Analysis

Cells were collected in lysis buffer containing protease and phosphatase inhibitors for protein isolation. We prepared cellular extracts by sonication. And total protein concentrations were determined for the Western blot analyses. Proteins were separated on 4–20% Tris-Glycine gels (Invitrogen, Carlsbad, CA, USA) and transferred to nitrocellulose membranes. After the membranes were blocked in TBST (10 mM Tris-HCl buffer, pH 8.0; 150 mM NaCl; and 0.1% Tween 20) and 5% (w/v) BSA at room temperature for 60 min, they were incubated overnight at 4°C with antigen-specific primary antibodies. The primary antibodies used were against phospho-NF- κ B p65 (Ser536) (Cell Signaling Technology, Danvers, MA, USA). The blots were under incubation with species-specific HRP-conjugated secondary antibodies for 2 hours at room temperature. And then, the proteins were visualized through incubation with a chemiluminescent substrate kit (Thermo Fisher Scientific, Waltham, MA, USA).

Statistics

Experiments were performed at least three times. The clinical and *in vitro* data were analyzed using SPSS 13.0 (Chicago, IL, USA). The results are expressed as means $\pm$ standard deviations. *P* value less than 0.05 was considered statistically significant.

Results

Laryngeal videostroboscopy image outcomes

The patients in this study had husky voices for more than 6 months. VFNs with mild to moderate phonatory gaps were seen under videostroboscopy. Figure 1 presents the bilateral vocal nodules at rest (Fig. 1A) and the glottal gap during phonation (Fig. 1B).

Patients were followed up 3 months after TCM treatment. Videostroboscopy, acoustic analysis, and perceptual assessment were conducted. Figure 1D and 1E presents the post-TCM glottal gap at rest and during phonation, respectively. We found that after TCM treatment, most patients achieved marked improvements in glottal closure, mucosal wave, and the amplitude of mucosal wave under videostroboscopy. No additional morbidities or complications were observed during treatment.

Objective and Subjective Voice Parameters

The acoustic analyses before and after TCM herb treatments are presented in Table 1. No significant differences were noted between MPT and NHR among the two groups ($P > 0.05$). Subjective VHI-C10 questionnaire scores, assessed by a blinded voice therapist, decreased significantly after TCM treatment.

Jitter, shimmer, and GRBAS scores also improved significantly. A significant decrease was also observed in pre- and post-TCM perceptual assessments, which were conducted according to total GRBAS scores (Table 2).

Table 1
Demographic data and pre- and post-TCM acoustic analysis results of study patients.

Results of acoustic analysis for patients enrolled in the present study			
Characteristics	Total patients (n = 22)		p value
	Pre	post	
Sex (Male/Female)	4/18		
Mean age	56 (32–75)		
Mean follow up time(d)	77 (12–150)		
MPT (sec)	9.02 ± 4.27	8.75 ± 4.52	0.770
Jitter	2.86 ± 1.77	1.80 ± 1.28	0.001*
Shimmer	0.67 ± 0.48	0.53 ± 0.50	0.003*
NHR	0.19 ± 0.10	0.16 ± 0.10	0.017*
VHI-C10	17.6 ± 8.06	6.6 ± 6.33	< 0.001*
GRBAS	5.91 ± 3.73	2.93 ± 3.34	< 0.001*
G	1.39 ± 0.74	0.84 ± 0.82	0.005
R	1.39 ± 0.74	0.75 ± 0.74	0.002
B	1.14 ± 0.77	0.57 ± 0.73	0.002
A	0.95 ± 0.84	0.36 ± 0.66	0.001
S	1.05 ± 0.84	0.41 ± 0.67	< 0.001

TCM Medication Review

We pooled the prescribed medications of the study participants and calculated the total doses. The following herbs were prescribed with total dosages of > 1500 g (Fig. 2): *P. grandiflorus*, licorice, *Ophiopogon japonicus*, *Fritillaria thunbergii*, and Sepiae Endoconcha (cuttlebone). The total prescribed amount of *P. grandifloras* and licorice are more than 2000 grams. The average duration and doses of *P. grandifloras* and licorice for the patients are summarized in Table 2.

Table 2
The duration and dosage for each patient of Licorice and *P. grandiflorus* obtained from a medication chart review of 22 patients.

	Licorice	<i>P. grandiflorus</i>
Duration (days)	63.95 ± 32.44	62.73 ± 32.89
Average		
Mean	69.5 (14–143)	63(14–143)
Dosage (grams)	97.09 ± 78.45	93.98 ± 79.73
Average		
Mean	68.5 (5.61–296)	60 (7.14–296)

P65 and mitogen-activated protein kinase Pathway Inhibition by *Glycyrrhiza glabra* and *Platycodon grandiflorus* in vitro

G. glabra and *P. grandiflorus* did not hinder the growth of PVFE cells even at high concentrations (Fig. 3A). In VFE cells treated with 50 ng/mL TNF α , NF- κ B p65, a downstream target of the mitogen-activated protein kinase (MAPK) pathways, was stimulated by phosphorylation. *G. glabra*, *P. grandiflorus* and the corticosteroid dexamethasone could reduce the expression of phosphorylated p65 in TNF α -treated cells (Fig. 3B). To further study the mechanism of the anti-inflammatory effect of TCM, the change in p-AMPK, p38, extracellular signal-regulated kinases (ERK), and Jun N-terminal kinases (JNK) in response to TCM treatment in VFE cells was investigated (Fig. 3C). *G. glabra* and *P. grandiflorus* significantly inhibited the p38, ERK, and JNK pathways, but their effect on the MAPK pathway was unclear.

Discussion

We collected the retrospective medical records of 22 vocal fold nodule patients treating with TCM from July 2018 to August 2020. We found that the most used herbs were *P. grandiflorus* and licorice. Further, we used the two herbs for porcine VFE culture. And the two herbs significantly inhibited the P38, ERK, and JNK pathways in TNF-alpha induced inflammation.

The finished herbal products (FHP) we prescribed are granulated compounds concentrated from Chinese herbal remedies, including single herbs and herbal formulae.(24) They are officially approved in the national healthcare systems not only in Taiwan, but also in Japan, Korea and mainland China. They are widely used in the East Asia. (25)

In our retrospective analysis, patients regularly return to clinic in a two-week to one-month manner. Since our clinic is comparatively remote, most of our patients found that returning to TCM clinic is more

convenient than attending voice therapy. From Fig. 2, we can see the most used herbs were Licorice and *P. grandifloras*. The total amount were more than 2000 grams.

Glycyrrhiza glabra, the licorice species we used here, is brought from Chiayi Chang Gung Memorial Hospital (Taiwan). *G. glabra* is grown in Eurasia, northern Africa and western Asia.(26) Its rhizomes and roots are the most important medicinal parts, which have been reported to be used alone or in combination with other herbs for treating multiple diseases such as digestive disorders, respiratory tract disorders, epilepsy, fever, sexual disability, and etc. (27) *P. grandiflorus* contains numerous amino acids, vitamins, and trace elements. Also, it has the ability to treat the following disorders: cough and asthma relief, antitumor activity, anti-inflammatory and antibacterial effects, antioxidation, hypoglycemic effects, liver protection, and immunity enhancement (28). Both *Glycyrrhiza glabra* and *P. grandiflorus* can be used as food and flavoring agents.

The combined use of these two herbs can be traced to ancient China. In the Chinese classic *Shang Han Lun* (English title: Treatise on Cold Damage Diseases, written by ZHANG Zhong-jing, 150–219 A.D.), the two herbs were combined in a 2:1 ratio and were used mainly to treat sore throat and Fei Yon (pulmonary abscess) (29, 30). Pharmacologically, they can affect the metabolic process of each other, improving the bioavailability of their compounds (31, 32).

Clinically, inflammation is commonly observed in patients with chronic voice disorders, such as vocal fold nodules. For example, an immediate increase in inflammatory cytokines, including TNF- α , is observed in response to vocal fold injury. The effect of TNF- α on the vocal fold epithelium upregulates mucin expression (16). TNF- α may activate the NF- κ B p65 pathway in PVFE cells, but this inflammatory pathway is inhibited by the corticosteroid dexamethasone(Dex) and high doses of *G. glabra* and *P. grandiflorus*. This means that the anti-inflammatory effect of TCM herbs on the vocal folds depends on their accumulation in vocal epithelium cells.

MAPK families are critical in complex cellular programs like proliferation, differentiation, development, transformation, and apoptosis. The MAPK pathways involve a series of protein kinase cascades, which play a crucial role in the regulation of cell proliferation. The three major MAPK families, namely mammalian ERKs, c-JNK, and p38 kinases, are activated by many stimuli. JNK and p38 are activated by cytokines and stressors, and ERK is mainly activated by growth factors (33). Figure 3C shows that *G. glabra* and *P. grandiflorus* deregulated the activation of p38, p-ERK, and p-JNK. This result may be explained by the beneficial effects of *G. glabra* and *P. grandiflorus* on vocal nodules due to downregulation of the MAPK pathway (34).

In our prescription pattern, a 1:1 ratio of *G. glabra* to *P. grandiflorus* may be more beneficial for VFN patients. Our sample size is relatively small. Also, the dosage and duration varied, which may be due to individual difference. For *in vitro* study, though the above inflammatory pathway down-regulation was seen, whether there exists synergistic effect or other interaction between the herbs are still unknown. Further prospective and functional studies are needed to confirm the results of this study and clarify the mechanism of action.

In conclusion, by reviewing clinical patient data and conducting an *in vitro* study, we confirmed that TCM herbal treatment is an alternative treatment option for VFNs. Patients' voices exhibited improvement after daily TCM herb use, and VFNs were reduced. From the above results, we may imply that our most prescribed herbs *G. glabra* and *P. grandiflorus* as potential VFN alternative treatment.

Abbreviations

VFN

vocal fold nodules

TCM

traditional Chinese medicine

VHI-C10

Chinese Voice Handicap Index-10

MDVP

multidimensional voice program

TNF-alpha

tumor necrosis factor- alpha

Dex

dexamethasone

PVFE

porcine vocal fold epithelial

GRBAS

G = grade, R = roughness, B = breathiness, A = asthenia, and S = strain

NHR

noise-harmonic ratio

MPT

maximum phonation time

AE

aqueous extracts

ERK

extracellular signal-regulated kinases

JNK

Jun N-terminal kinases

MAPK

mitogen-activated protein kinase

FHP

finished herbal products

Declarations

Availability of data and materials

Not applicable

Ethics approval and consent to participate

The present study was approved by the Institutional Review Board of Chiayi Chang Gung Memorial Hospital (No.201901034B0). All participants gave written informed consent prior to the beginning of the study.

Consent for publication

Not applicable

Competing interests

The authors declare that they have no competing interests.

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Authors' Contributions

Conceptualization, C.-M.H. and C.-Y. W.; methodology, M.-Y.Y.; formal analysis, S.-F.C., M.-S.T. and Y.-H.Y.; writing—original draft preparation, C.-M.H. and I.-Y.L.; writing—review and editing, M.-Y.Y., G.-H.C. P.-R.Y. and Y.-T.T.; supervision, C.-M.H.; project administration, C.-M.H.. All authors have read and agreed to the published version of the manuscript.

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Figures

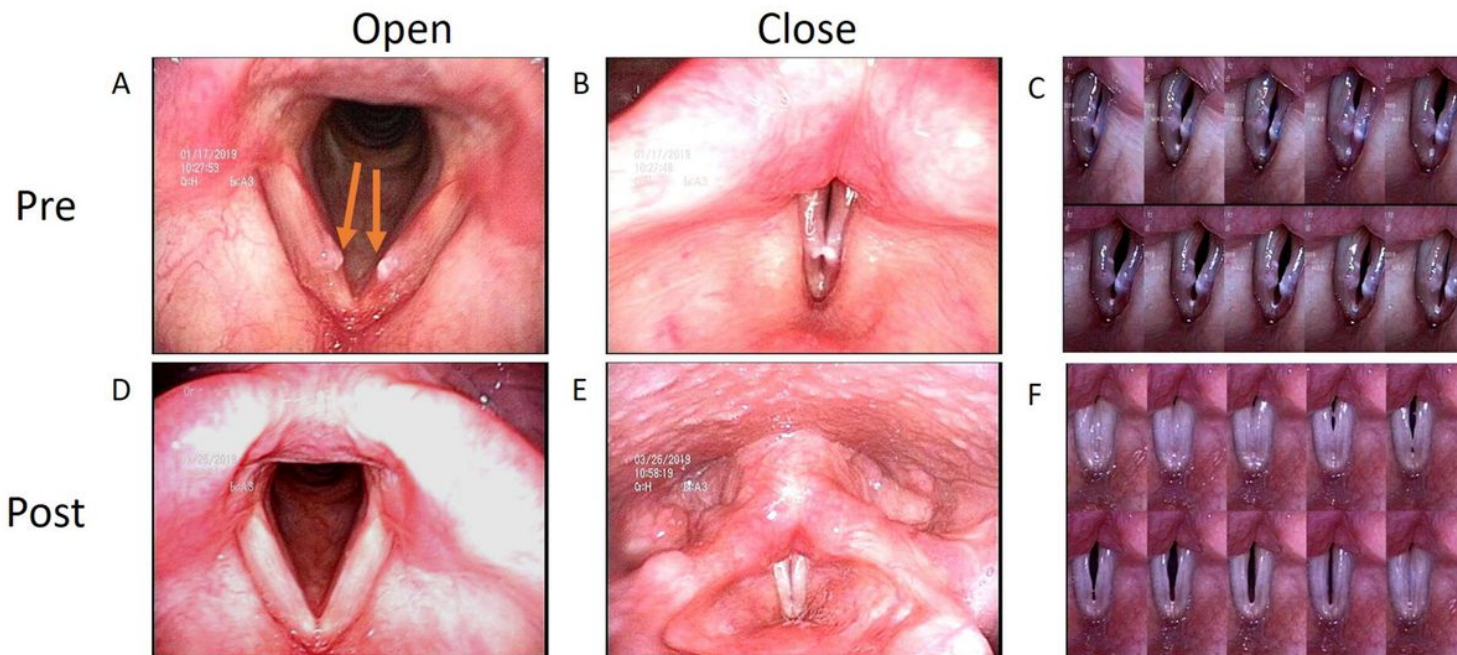


Figure 1

Videostroboscopy record of a 42-year-old female patient before and after 2-week TCM treatment. (A) bilateral vocal nodule before TCM (at rest). (B) Glottal gap (phonation). (C) Persistent glottal gap in montage photograph during phonation. (D) No vocal nodule after TCM (rest). (E) No glottal gap (phonation) (F) Montage photograph of normal glottal gap in during phonation.

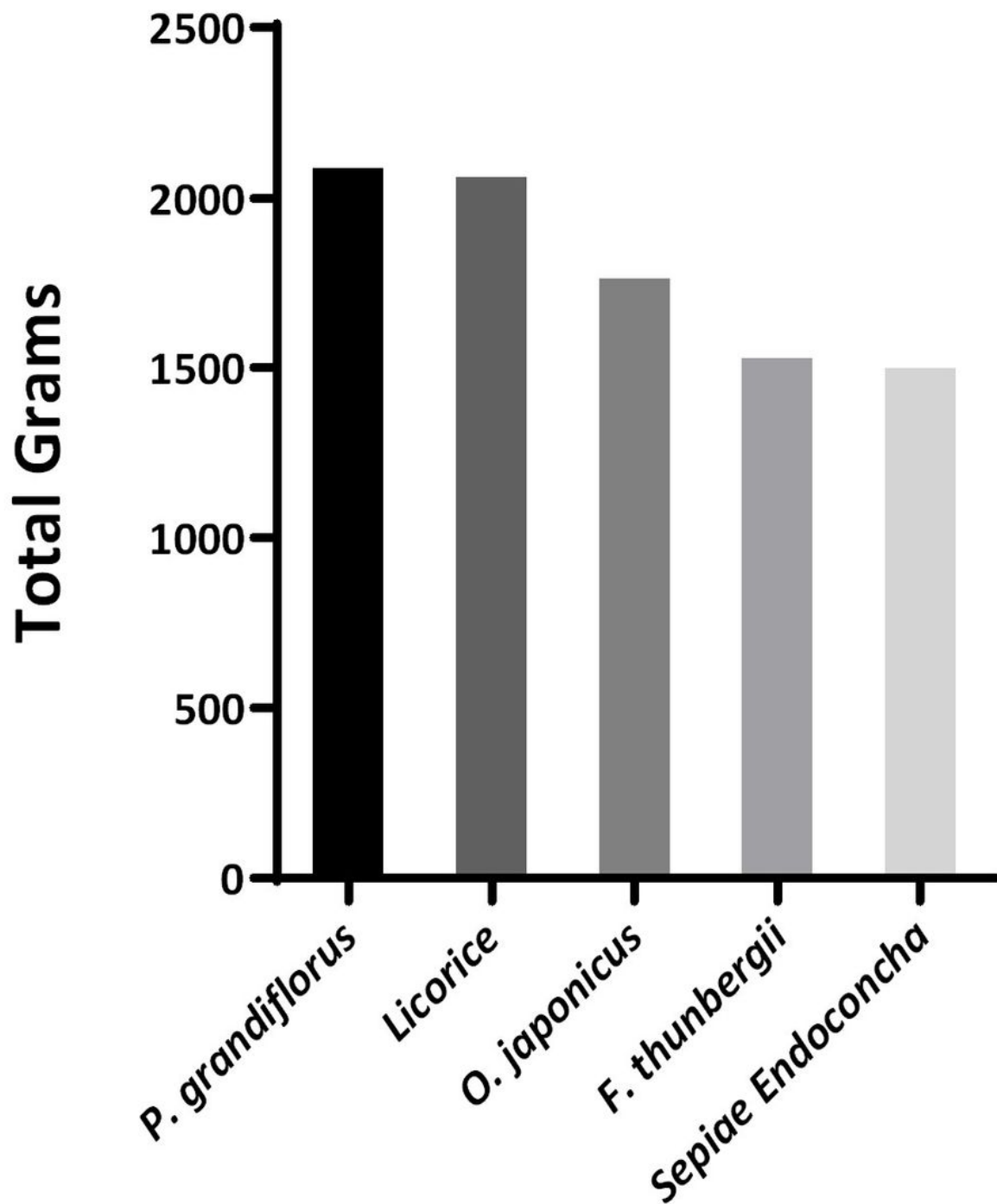


Figure 2

Total dosage ranking of the medication prescribed to 22 patients.

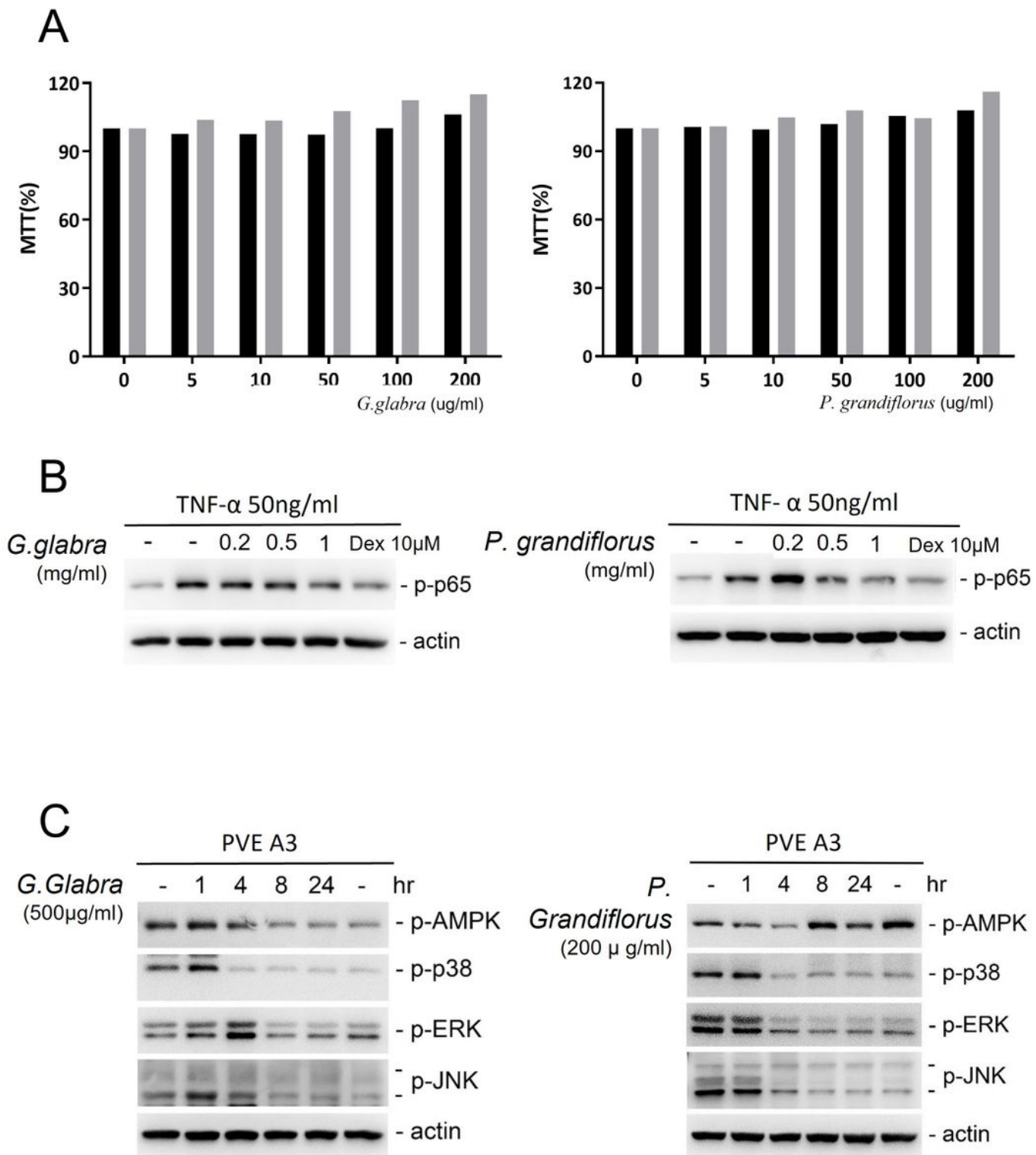


Figure 3

PVFE cells were treated with Glycyrrhiza glabra and Platycodon grandiflorus. (A) *G. glabra* and *P. grandiflorus* did not result in cell toxicity in PVFE cells. (B) *G. glabra*, *P. grandiflorus* and dexamethasone (Dex) reduced the expression of phosphorylated p65 in TNF α -treated cells. (C) P38, ERK, and JNK pathways were inhibited by *G. glabra* and *P. grandiflorus*, but their effect on the MAPK pathway was unclear.