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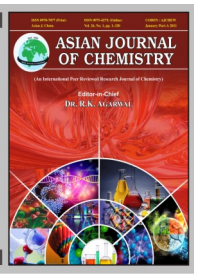

Preparation and Immunological Evaluation of Organic Acid Salts of Levamisole

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Four kinds of organic acid salts of levamisole were prepared from levamisole hydrochloride and organic acids (L-ascorbic acid, tartaric acid, citric acid and ferulic acid) by solvent crystallization method, respectively. The salts were characterized by elemental analysis, UV-VIS, FTIR, ESI-MS, DSC-TGA, solubility in water, melting point, optical rotation and pH in water. The immunosuppressed mice models were established by intraperitoneal injection of cyclophosphamide to verify and compare the effects of levamisole hydrochloride and organic acid salts of levamisole on immune functions of immunosuppressed mice. The results showed while significantly increasing the contents of serum IgM and IgG and the immune organ indexes in cyclophosphamide-induced immunosuppressed mice, levamisole ferulate and levamisole tartrate presented higher increases in IgG contents and immune organ indexes than other salts did. Thus, it could be concluded that levamisole ferulate and levamisole tartrate were more effective on immune enhancement than other salts.

Keywords: Levamisole, Salt formation, Organic acid salts, Immune enhancement.

INTRODUCTION

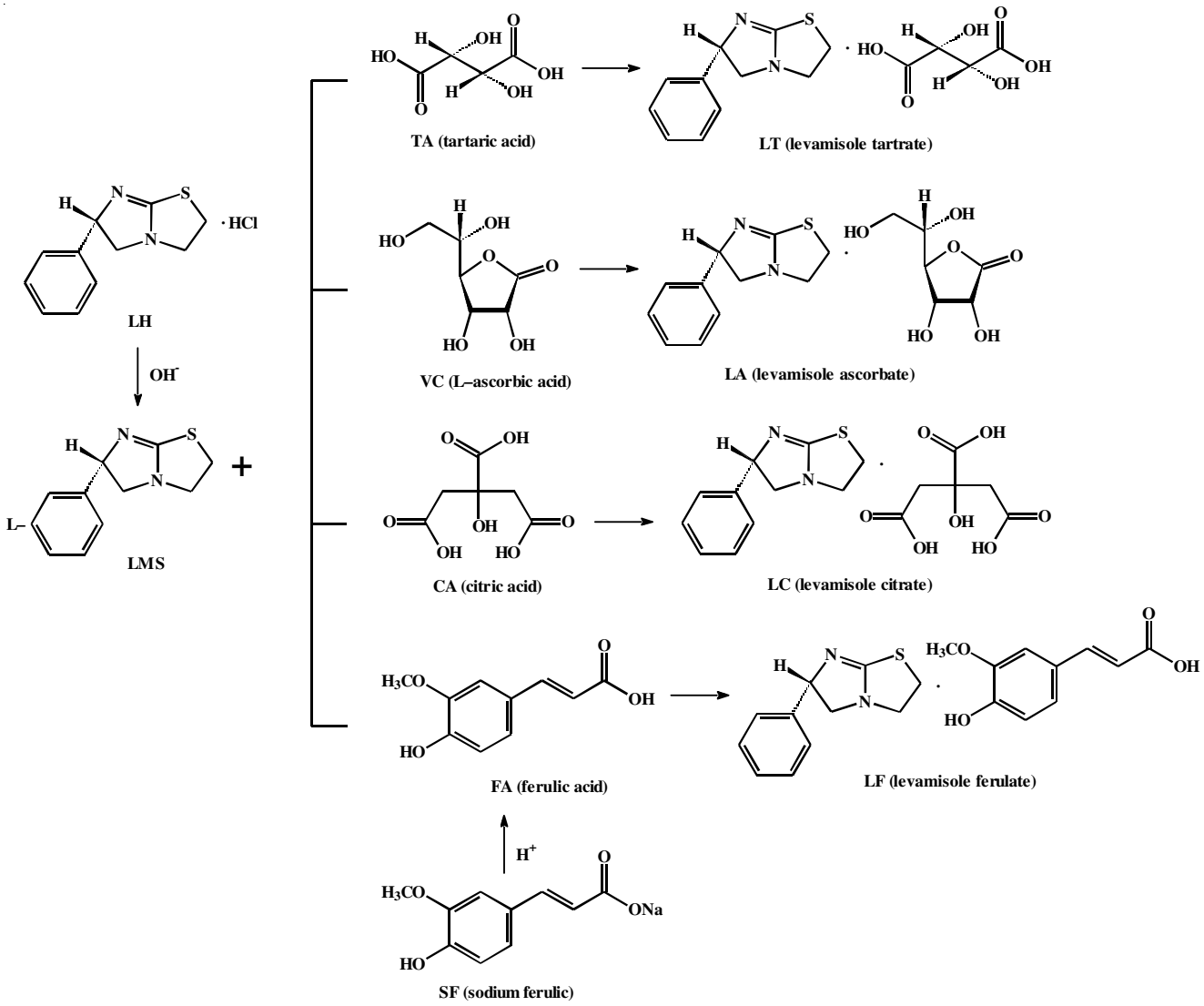
Levamisole (LMS), the levo-isomer of tetramisole, is an anthelminthic belonging to a class of synthetic imidazothiazole derivatives, as well as an immunomodulator which can enhance the immune responses[1-5]. Due to the poor solubility and instability in water, its hydrochloride (levamisole hydrochloride, LH) is commonly used in clinical. However, LH still has problems of low bioavailability and side effects, such as a certain stimulus to the human body. In the past decade, a large number of researches have been increasingly reported with respect to chemical modifications of drugs, such as esterification[6], acylation[7], amidation[8], alkylation[9], anhydride formation[10], salt formation[11-17], ring opening and formation[18,19]. As reported, chemical modifications have many advantages such as improving the solubility, stability and bioavailability of drugs, reducing toxic reactions, enhancing pharmacological effects, extending drug action, making other drug dosage forms and delivery systems possible or more excellent, *etc*.

Salt formation is a simple and effective chemical modification, which can influence a number of physicochemical properties of the parent drug, including solubility, dissolution rate, stability and hygroscopicity[11]. As a result, different salts of drugs may bring about different pharmacokinetic and pharmacodynamic qualities and therefore different clinical qualities; some even may show intrinsic toxicity[14]. Levamisole base has a tertiary nitrogen atom, whose lone pair of electrons can accept a proton, so it's likely to form stable salts with acids. To the best of our knowledge there has been no report on the use of organic acids for the synthesis of the salt form of levamisole. In this study, a series of organic acid salts of levamisole were prepared (**Scheme-I**) and characterized. Meanwhile, immunological evaluation of the five salts [evamisole hydrochloride (LH), levamisole ascorbate (LA), levamisole tartrate (LT), levamisole citrate (LC) and levamisole ferulate (LF)] was carried out on cyclophosphamide (CTX)-induced immunosuppressed mice models. The immune organ indexes and contents of serum IgA, IgG and IgM were measured and compared in an attempt to find and obtain a more effective salt form of levamisole on immune enhancement.

EXPERIMENTAL

Levamisole hydrochloride (Shaanxi Hanjiang Pharmaceutical Co., Ltd., batch number: 090610); cyclophosphamide (Jiangsu Hengrui Medicine Co., Ltd., batch number: 090802); D-tartaric acid (TA) (analytical grade, Nantong Qihai Chemical Co., Ltd.); citric acid (CA) (analytical grade, Shanghai Pan Ke Biotechnology Co., Ltd.); ferulic sodium (Livzon Pharmaceutical Group Inc., batch number: 080307); L-ascorbic acid (VC) (analytical grade, Chongqing Boyi Chemical Co., Ltd.); IgG, IgM and IgA kits (Adlitteram Diagnostic Laboratories Inc.); Other reagents were commercially available analytical grade.

Preparation of organic acid salts of levamisole: Levamisole hydrochloride (12 g, 0.05 mol) was dissolved in



Scheme-I: General synthesis of organic acid salts of levamisole

50 mL of distilled water under the condition of ice bath. With magnetically stirring, 50 mL of 1 M NaOH solution was dropwise added to the LH solution until the pH became 7 and white turbidity appeared. Dropwise adding was continued until the pH finally became 12. Following warming the reaction system to 40 ºC in a water bath, stirring was continued for another 0.5 h while maintaining the pH at 12. Then, the resultant mixture was filtered to separate levamisole base from the system. The first part of product was washed to neutral pH with distilled water and dried under reduced pressure. The filtrate was extracted three times with dichloromethane. The extract was dried overnight in anhydrous calcium chloride and filtered. The resultant filtrate was evaporated to remove dichloromethane under reduced pressure. The second part of levamisole base was obtained and combined with the first part. The total yield was 98.32 %.

All the four salts LF, LA, LC and LT were prepared by solvent crystallization method as follows: Levamisole base and respective organic acids (mole ratio 1:1) were dissolved in absolute ethanol and stirred for a period of time at a suitable temperature. The reaction mixture was filtered. The filtrate was chilled to 5-10 ºC, followed by slowly adding an appropriate amount of anhydrous ether and freezing crystallization for 1h. The resultant mixture was filtered. The filter cake was washed with a small amount of anhydrous ether and dried in phosphorus pentoxide. The crude product was recrystallized with anhydrous methanol-ethyl acetate to obtain more pure organic acid salts of levamisole.

Characterization of organic acid salts of levamisole: The solubility in water at 25 ºC (GB/T 21845-2008, China), melting point (MP), optical rotation ($[\alpha]_D^{20}$) and pH in water were tested. A UV-VIS spectrophotometer (UV-2450, Shimadzu, Japan) was used for the spectra measurements of LMS, LH, LA, LT, LC, LF and the four organic acids in ethanol. Elemental Analysis was determined by using an elemental analyzer (CE-440 Elemental Analyzer, EAI, USA). Fourier transform infrared spectra of LMS, LH, LA, LT, LC, LF, TA, VC, CA, ferulic acid (FA) and four physical mixtures (LMS and VC, LMS and TA, LMS and CA, LMS and FA) were obtained in the range of 4000-400 cm^{-1} using a fourier transform infrared spectrometer (Spectrum GX, Perkin-Elmer, USA), according to the KBr disk method. Electrospray ionization mass spectrometry (ESI-MS) was carried out on a mass spectrometer (micrOTOF Q, Bruker, Germany) equipped with an electrospray ionization

source. LA, LT, LC and LF were dissolved in methanol and diluted to use, respectively. Infusion rates were 3 μL min^{-1} during sample analysis. DSC and TGA curves of LMS, LH, LA, LT, LC and LF were recorded on a differential scanning calorimeter (STA-449C, NETZSCH, Germany). All the samples were accurately weighed (2-5 mg), put in aluminium pans and heated at a scanning rate of 10 ºC min^{-1} from 25-400 ºC. Nitrogen was used as the purge gas with the flow rate set at 30 mL min^{-1}. An identical empty pan was used as a reference.

Immunological evaluation: Seventy adult *Kunming* mice (35 males and 35 females) of clean grade, weighing 19-23 g, were obtained from the experimental animal center of Chongqing Academy of Animal Sciences. All experimental procedures were in accordance with the ethics and regulation of animal experiments of Chongqing Academy of Animal Sciences. The mice were housed in cages at a temperature between 20-25 ºC and relative humidity of 40-60 % under natural light/dark conditions for 1 week and were given conventional feed and free drinking water.

Seventy mice were randomly divided into 7 groups of 5 males and 5 females each. One group was intraperitoneally injected with saline solution and the remaining groups were injected with cyclophosphamide (CTX) solution (50 mg kg^{-1} d^{-1}) for five consecutive days to establish immunosuppressed models. From the sixth day, five immunosuppressed groups were intraperitoneally injected with equimolar LH, LA, LT, LC and LF solutions (12, 19, 17.7, 19.8 and 20 mg kg^{-1} d^{-1}) for 21 consecutive days, respectively, while other groups were injected with an equivalent volume of saline solution. The resultant groups were named LH-CTX group, LA-CTX group, LT-CTX group, LC-CTX group, LF-CTX group, CTX group and Control group, respectively. Body weights were measured on time every day, while recording the clinical performances of all groups. Following the last administration, all mice were fasted for 12 h, but had free access to fresh water. Then whole blood samples were collected in Eppendorf tubes (SorensonTM, Bio Science, Inc. USA) from the orbits and allowed to clot for at least 1 h at 4 ºC. Samples were centrifuged at 4 ºC (10 min, 3000 × g) and the serum was separated and stored at -20 ºC until further analysis.

Determination of serum antibody content: An enzyme linked immunosorbent assay (ELISA) was carried out to measure the contents of serum IgG, IgM and IgA using a microplate reader (ELX808, Baote, USA), according to the kit instruction (Adlitteram Diagnostic Laboratories Inc.).

Determination of immune organ indexes: The mice were killed by cervical dislocation and weighed. Organs (thymus, spleen and liver) were obtained and washed with distilled water, removing the connective tissue and fat on their surfaces. The organs were weighed after sucking up all the water with filter paper and observed under a microscope (Shanghai Yuguang Instrument Co., Ltd.). At last, the immune organ indexes were calculated using the following equation[20]:

$$\text{Immune organ (thymus, spleen or liver) index (mg g}^{-1}) = \frac{\text{Weight of thymus, spleen or liver (mg)}}{\text{Body weight of the mouse (g)}}$$

Statistical method: All data were statistically analyzed by SPSS17.0 software. All results were presented as mean ± standard deviation ($\bar{x} \pm s$). Comparisons between groups were carried out using one-way ANOVA, wherein Least-Significant Difference (LSD) method was used for homogeneity of variance and Tamhane's T2 method was used for herterogeneity of variance. The differences were statistically significant when $p < 0.05$.

RESULTS AND DISCUSSION

Based on advantages of salt formation and combination principles in pharmaceutical chemistry that structures of two compounds can be put together in one molecule for the purpose of reducing poisonous side effects and obtaining the joint effect, four organic acid salts of LMS (LA, LT, LC and LF) were prepared in order to find a more effective salt form of enhancing immune function.

Characterization of organic acid salts of levamisole: Four organic acid salts of levamisole were obtained. Levanisole ascorbate was a light yellow crystalline powder without odor and bitter taste. Levanisole tartarate and LC were white crystalline powders without odor and bitter taste, but LF was a light green crystalline powder with bitter taste and odorless. They were all soluble in water, the solubility results are listed in Table-1. Levanisole ascorbate solution was readily oxidized to turn dark yellow, whereas other three solutions were more stable. LA, LT and LC were dissociated into white insoluble precipitate in 0.1 M NaOH solution, which was identified as LMS by thin layer chromatography method. Levanisole ferulate was dissociated into white insoluble precipitates both in 0.1 M HCl solution and 0.1M NaOH solution, which were identified as FA and LMS by TLC, respectively. Meanwhile, elemental analysis was tested, as well as some other physical properties, including melting point, optical rotation ($[\alpha]_D^{20}$) and pH, which were different from those of LMS (Table-1). The results of elemental analysis showed that the measured values were in good accordance with the calculated ones for the C, H and N contents of organic acid salts of levamisole.

TABLE-1
RESULTS OF ELEMENTAL ANALYSIS AND SOME PHYSICAL PROPERTIES OF ORGANIC ACID SALTS OF LEVAMISOLE

Compounds	Solubility in water at 25 ºC (mg/mL)[b]	m.p. (ºC)	$[\alpha]_D^{20}$	pH (H_2O)	Element content (%)[a]					
					C		H		N	
					Meas.	Count	Meas.	Count	Meas.	Count
LA	137.19 ± 1.17	175.2-176.9	-23.34	5.93	53.59	53.67	5.36	5.30	7.42	7.36
LT	151.95 ± 0.44	163.6-165.1	-141.68	5.86	50.73	50.84	5.28	5.12	7.85	7.91
LC	184.47 ± 0.48	125.4-126.7	-116.52	5.52	51.67	51.51	5.14	5.09	6.93	7.07
LF	105.25 ± 0.80	141.6-142.7	-98.01	6.15	63.21	63.30	5.48	5.56	7.15	7.03

[a]Meas. indicates the measured values, count indicates the calculated ones. [b]Solubility is present as Mean ± standard deviation, n = 3.

Thus, it may be concluded that levamisole reacted with organic acids to form salts.

UV Spectroscopic analysis: UV spectroscopy was used to observe the behaviour of the LMS absorption spectra with the addition of different organic acids. The UV spectra of LMS, VC, FA, LA, LT, LC and LF are shown in Fig. 1. LMS, LC, LT, VC and FA exhibited absorption peaks at 208, 214, 214, 265 and 322 nm, respectively. Levanisole ascorbate exhibited two absorption peaks at 212 and 254 nm. Levanisole ferulate exhibited two absorption peaks at 212 and 315 nm. However, TA and CA had no obvious absorption in the wavelength range of 200-400 nm. As shown in Fig. 1, the absorption peak of LMS at 208 nm shifted to 214 nm (absorption peaks of LC and LT), 212 nm (one absorption peak of LA and the absorption peak of LF), respectively. The absorption peak of VC at 265 nm and the absorption peak of FA at 322 nm had hypsochromic shifts to 254 nm (one absorption peak of LA) and 315 nm (one absorption peak of LF), respectively. In addition, the peak shapes of LA, LT, LC and LF were distinguished from those of LMS and organic acids, as well as those of their simple summations, respectively. From the above, it could be concluded that the shifts of absorption peaks and the changes of peak shapes were due to the formation of organic acid salts of levamisole.

Mass spectrometry (ESI-MS): The main ions fragments of mass spectrometry are listed in Table-2. The ions corresponding to LMS (*m/z* 205), VC (*m/z* 175), TA (*m/z* 149), CA (*m/z* 191), FA (*m/z* 193) were seen as strong peaks in the scan spectra of respective solutions of organic acid salts of levamisole, indicating that the bonds connecting LMS and organic acids were weak so that organic acid salts of LMS were readily subjected to cleavage to form the ions of LMS and organic acids. The scan spectra also showed peaks at *m/z* 379, 353, 395 and 397 ions corresponding to the 1:1 compounds of LMS with VC, TA, CA and FA, respectively. All these results suggested the formation of organic acid salts of levamisole.

Fourier transform infrared spectroscopy: FTIR spectroscopy was also used to detect the possible molecular interaction between LMS and organic acids, since upon the formation of organic acid salts of LMS shifts or changes in the absorption spectrum occured. FTIR spectra of all samples are illustrated in Fig. 2. Characteristic absorption peaks of LMS such as C=N and C-S stretching vibrations (1465 and 874 cm^{-1}) were observed in the spectra of the four salts. However, there were some changes that functional groups showed band shifts, broadening and disappearing in the FTIR spectra of LA, LT, LC and LF when compared to those of LMS, VC, TA,

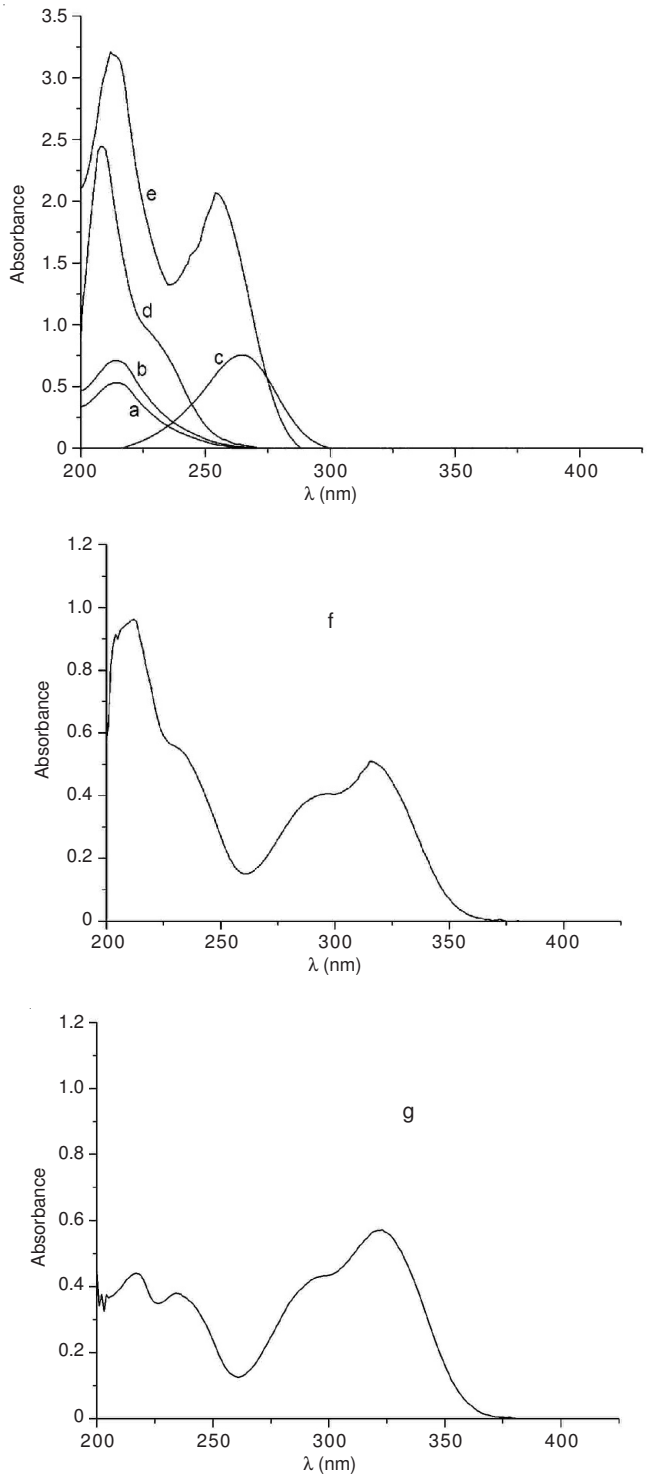


Fig. 1. UV spectra of LC (a), LT (b), VC (c), LMS (d), LA (e), LF (f) and FA (g)

TABLE-2
RESULTS OF MASS SPECTROMETRY

Compounds	Molecular formula	Molecular weight	Ionization mode	Peak(m/z)
LA	$C_{11}H_{12}N_2S.C_6H_8O_6$	380	(Positive) ESI	205[LMS+H]$^+$ 177[VC+H]$^+$
			(Negative) ESI	175[VC-H]$^-$ 379[LA-H]$^-$ 396[2VC-2H+Na]$^-$
LT	$C_{11}H_{12}N_2S.C_4H_6O_6$	354	(Positive) ESI	205[LMS+H]$^+$ 173[TA+Na]$^+$
			(Negative) ESI	149[TA-H]$^-$ 353[LT-H]$^-$
LC	$C_{11}H_{12}N_2S.C_6H_8O_7$	396	(Positive) ESI	205[LMS+H]$^+$ 215[CA+Na]$^+$
			(Negative) ESI	191[CA-H]$^-$ 395[LC-H]$^-$
LF	$C_{11}H_{12}N_2S.C_{10}H_{10}O_4$	398	(Positive) ESI	205[LMS+H]$^+$ 217[FA+Na]$^+$
			(Negative) ESI	193[FA-H]$^-$ 397[LF-H]$^-$

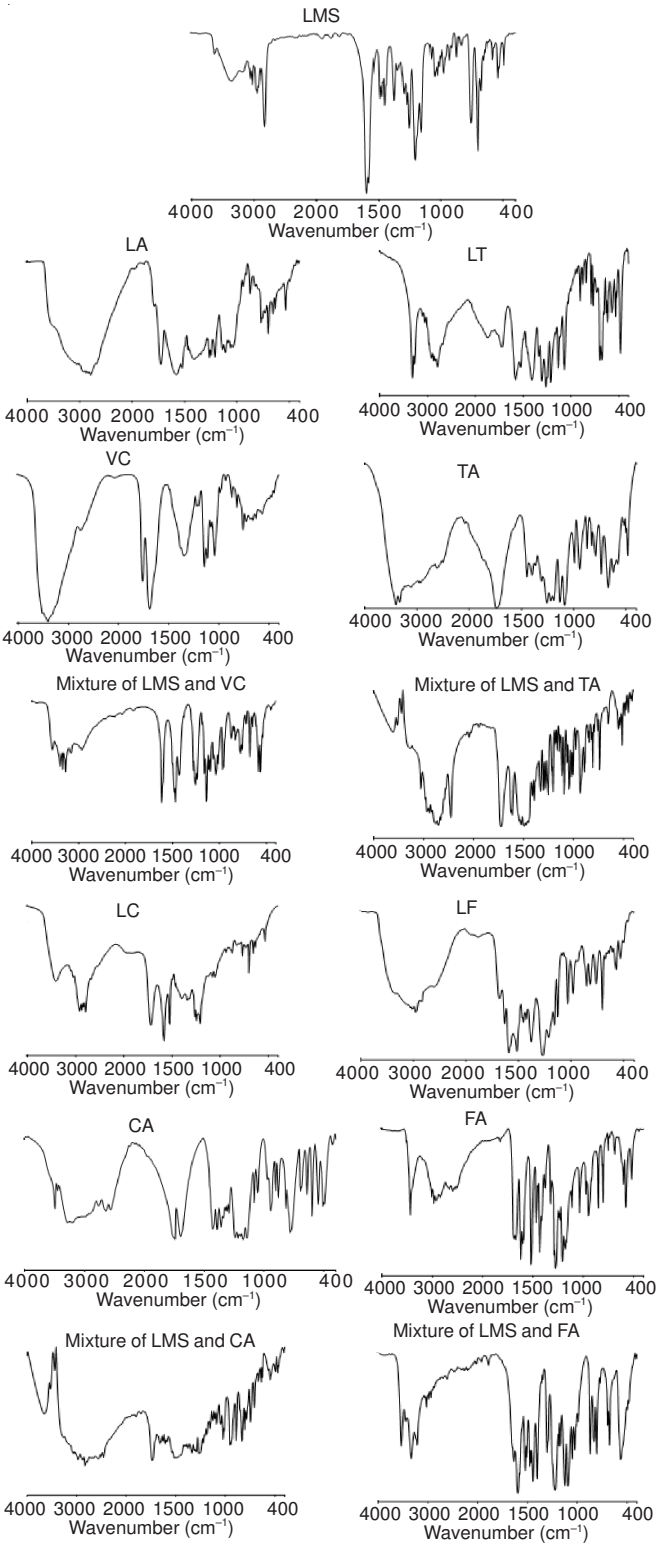


Fig. 2. FTIR spectra of all samples

CA, FA and their respective physical mixtures. The FTIR spectrum of LA displayed a broad absorption band at 3420 cm^{-1}, three O-H stretching vibrations at 3614, 3321 and 3037 cm^{-1} and two C-O stretching vibrations at 1062 and 1032 cm^{-1}. Levanisole tartarate showed peaks at 3071 cm^{-1} ν(O-H), 1759 cm^{-1} ν(C=O), 1248 cm^{-1} ν(C-O), 1350 cm^{-1} β(O-H) and 1101 cm^{-1} ν(C-O), which could be attributed to the carboxyl group and secondary alcohol hydroxyl group. Levanisole citrate presented peaks at 1363 cm^{-1} β(O-H), 1136 cm^{-1} ν(C-O), 1263 cm^{-1} ν(C-O), 1583 cm^{-1} ν_{as}(C-O) and 1529 cm^{-1} ν_{s}(C-O) and its weak absorption band in the range of 3000-2500 cm^{-1} disappeared, which was the characteristic band of the carboxyl group of CA. Levanisole ferulate showed peaks at 1594, 853 and 821 cm^{-1}, which could be attributed to the oxygen anion of carboxyl and the *para*-substitution and *meta*-substitution of benzene ring, respectively, but its broad absorption band between 3400-2500 cm^{-1} ν(O-H) disappeared, which could be observed in the FTIR spectra of FA. Conclusively, FTIR spectra obtained from various LMS organic acid salts showed peaks which were not a summation of the characteristic peaks of LMS and organic acids and thus couldn't be simply regarded as the superposition of FTIR spectra of LMS and organic acids, indicating the interaction of LMS with organic acids.

DSC and TGA analyses: DSC can be used to investigate thermal events such as physical transitions (the glass transition, crystallization, melting and the vaporization of volatile compounds) and chemical reactions. The information obtained characterizes the sample with regard to its thermal behaviour and composition. TGA is used to measure the change in mass of a sample as a function of temperature or time. It provides information on the properties of the sample and its composition. If the sample decomposes as a result of a chemical reaction, the mass of the sample often changes in a stepwise fashion. The temperature at which the step occurs characterizes the stability of the sample material in the atmosphere used. For more information, simultaneous DSC is often used to measure with TGA[21].

Fig. 3 shows the DSC and TGA curves obtained for LT, LA, LC, LF and LMS. The curves of LT and LC presented endothermic peaks at 165 and 127 ºC corresponding to respective melting points and exothermic peaks at 235 and 250 ºC, respectively. The curve of LA presented a sharp endothermic peak at 176 ºC corresponding to the melting point and two consecutive exothermic peaks at 210 and 265 ºC caused by the oxidative decomposition of LA. The curve of LF presented a sharp endothermic peak at 142 ºC corresponding to the melting point and an exothermic peak at 250 ºC. But the curve of LMS presented a sharp endothermic peak at 61 ºC and an exothermic peak at 263 ºC corresponding to the melting and decomposition of LMS, associated with loss of moisture between 25 and 100 ºC. Apparently, the melting points of LMS organic acid salts significantly increased, indicating that new compounds had been formed.

In the TGA curves shown in Fig. 3, there was no weight loss due to water evaporation for LA, LT, LC and LF, but a small weight loss for LMS. LT began to decompose before melting. Other salts began to decompose in an exothermic process after melting. This could be seen in the TGA curves as a weight loss as well as in the DSC curves as exothermic peak decomposition. Meanwhile, the process of weight loss of LA, LT, LC and LF was differentiated from that of LMS by the combination of TGA and DSC curves, which was another indication of the formation of organic acid salts of LMS.

Immunological evaluation: Since products for immune enhancement are mainly used by the population with low immunity at the present, the immunological evaluation performed on immunosuppressed animal models is reasonable and

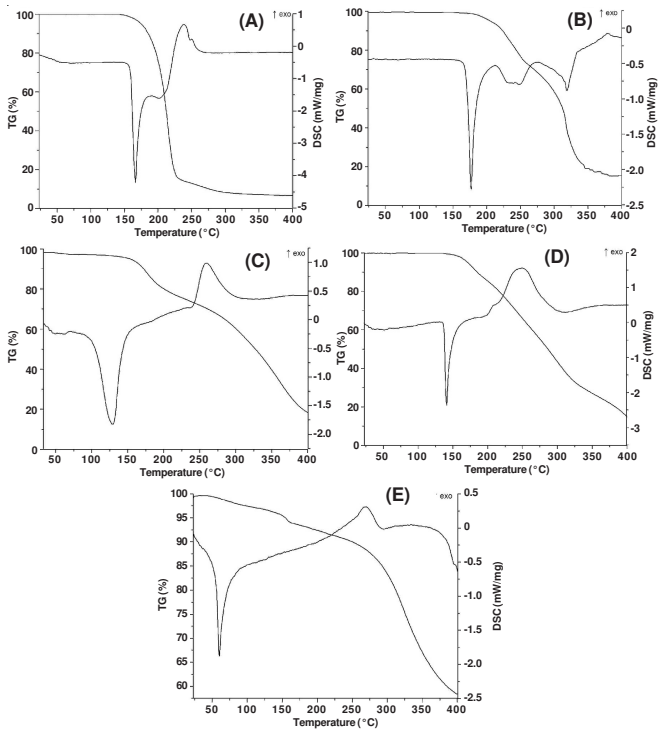


Fig. 3. TGA-DSC curves of LT (A), LA (B), LC (C), LF (D) and LMS (E)

effective. Without activity *in vitro*, CTX is one of the most commonly used drugs in the clinical treatment of tumors, which can be hydrolyzed to glyciphosphoramide with activation by excess ifosfamide or phosphatase *in vivo* and thus suppress both humoral and cell-mediated immune responses[22]. Therefore, it has been commonly used to establish immunosuppressed animal models[23,24].

Determination of serum antibody content: Immunoglobulins play a major role in humoral immune responses. Serum IgG, IgA and IgM are the molecular basis of humoral immune responsesplaying a role in specific immunity responses. In addition, IgG and IgM can activate the classical pathway of complement to take part in non-specific immunity responses[25].

Table-3 shows that the serum IgG and IgM contents of CTX group were significantly lower than that of Control group ($p < 0.05$), but the IgA content between them had no significant difference, indicating that CTX were able to suppress the generation of IgG and IgM effectively and had no obvious influence on the generation of IgA in mice. This corresponded to the report that CTX could inhibit both humoral and cell-mediated immune responses[22], giving information that the models established in this study were successful.

TABLE-3
CONTENTS OF SERUM IgA, IgG AND IgM OF MICE IN DIFFERENT GROUPS [n = 10, $(\bar{x} \pm s)$][a]

Group	IgA (mg/mL)	IgG (mg/mL)	IgM (mg/mL)
Control	1.04 ± 0.14a	9.47 ± 1.73de	0.54 ± 0.07cd
CTX	0.94 ± 0.16a	5.83 ± 1.43a	0.35 ± 0.08a
LH-CTX	0.98 ± 0.10a	9.13 ± 1.75cde	0.47 ± 0.12bc
LT-CTX	0.93 ± 0.13a	10.17 ± 0.93ef	0.56 ± 0.06d
LA-CTX	0.95 ± 0.16a	7.92 ± 1.28bc	0.51 ± 0.08bcd
LC-CTX	0.95 ± 0.10a	7.46 ± 1.51b	0.53 ± 0.06cd
LF-CTX	0.99 ± 0.18a	10.79 ± 1.05f	0.58 ± 0.07d

[a]Different small letters in the same column indicate significant differences ($p < 0.05$).

As shown in Table-3, serum IgA contents of all groups were not significantly different from each other ($p > 0.05$), but all salts significantly increased the contents of IgG and IgM in the serum of immunosuppressed mice ($p < 0.05$). Moreover, serum IgM contents of all groups for levamisole salts showed no significant difference when compared to that of Control group. The serum IgG content of LF-CTX group was significantly higher than those of Control group, LH-CTX group, LA-CTX group and LC-CTX group ($p < 0.05$), while the serum IgG content of LT-CTX group was only higher than those of LA-CTX group and LC-CTX group ($p < 0.05$). This indicated LF may be more effective on improving the antibody level *in vivo*, implying that LF could convert to LMS and FA to produce a joint effect *in vivo* because FA is an immunopotentiator[26] as well as LMS. However, for LF and LT there was no significant difference between their IgG and IgM contents ($p > 0.05$).

Determination of immune organ indexes: The results of body weights of all groups are listed in Table-4. Body weights of all groups increased in varying degrees, wherein CTX group gained the least amount of weight. The body weights of LT-CTX group, LF-CTX group and control group significantly increased when compared to those of other groups ($p < 0.05$). The LH-CTX group and CTX group became dispirited and inactive after injection. However, other groups were different, wherein especially LT-CTX group and LF-CTX group with injection after 0.5 h became inactive firstly, but returned to normal after 0.5 h of excitement and gained weight gradually.

In recent years, immune organ indexes have been commonly applied to the evaluation of immunomodulators on immune function, which to some extent can reflect the number and function of immune cells[27]. The results for immune organ indexes are presented in Table-4. Compared with control group,

TABLE-4
BODY WEIGHT AND THYMUS, SPLEEN, LIVER INDEXES OF MICE IN DIFFERENT GROUPS [n = 10, $(\bar{x} \pm s)$][a]

Group	Body weight (g)		Body weight change (g)	Index (mg g^{-1})		
	Before experiment	After experiment		Thymus index	Spleen index	Liver index
Control	21.50 ± 1.21	27.79 ± 1.00	6.29 ± 1.70c	4.62 ± 0.29d	6.44 ± 0.30c	58.60 ± 4.24ab
CTX	21.30 ± 1.12	24.76 ± 0.98	3.45 ± 1.69a	2.88 ± 0.55a	4.95 ± 0.41a	57.85 ± 3.87ab
LA-CTX	20.97 ± 1.12	24.89 ± 1.18	3.93 ± 0.68a	3.66 ± 0.53b	4.86 ± 0.56a	60.04 ± 4.31b
LT-CTX	20.77 ± 1.26	26.49 ± 0.91	5.72 ± 1.20cd	4.31 ± 0.43cd	6.06 ± 0.44c	58.90 ± 2.09ab
LC-CTX	20.49 ± 0.90	24.54 ± 1.03	4.05 ± 1.23a	3.54 ± 0.57b	5.25 ± 0.47b	60.07 ± 2.12b
LF-CTX	21.73 ± 0.63	27.26 ± 1.40	5.53 ± 1.73bc	4.56 ± 0.22d	6.45 ± 0.40c	58.72 ± 2.25ab
LH-CTX	20.85 ± 1.30	24.83 ± 0.79	3.98 ± 0.94a	3.03 ± 0.37a	5.57 ± 0.28b	59.54 ± 2.10b

[a]Different small letters in the same column indicate significant differences ($p < 0.05$).

the thymus and spleen indexes of CTX group significantly decreased ($p < 0.05$), while the decrease of the liver index was not significant ($p > 0.05$), suggesting both the thymus and spleen had atrophied and thus probably couldn't perform immune function normally because of injection of CTX. As shown in Table-4, liver indexes showed no significant difference between any two groups. The thymus indexes of LA-CTX group, LT-CTX group, LC-CTX group and LF-CTX group showed significant increases, compared with that of CTX group ($p < 0.05$). The spleen indexes of LH-CTX group, LC-CTX group, LT-CTX group and LF-CTX group were significantly higher than that of CTX group ($p < 0.05$). However, both the spleen and thymus indexes of LT-CTX group and LF-CTX group showed no significant difference when compared with those of control group ($p > 0.05$), whereas they were significantly higher than the spleen and thymus indexes of the remaining groups, indicating LF and LT were more effective on restoration of immune organs than other salts, which may be attributed to their higer bioavailablity. Furthermore, the spleen and thymus indexes of LF-CTX group were not significantly different from those of LT-CTX group ($p > 0.05$), which was in accordance with the above result for their serum antiboby contents, suggesting that LF and LT had similar effects on immune enhancement. However, studies on bioavailablity are necessary to further confirm the results, because demonstration of bioequivalence is generally the most appropriate method of substantiating therapeutic equivalence between medicinal products which are pharmaceutically equivalent or pharmaceutical alternatives[28].

Except for an immunomodulator, (S)-levamisole hydrochloride was recently shown to be an inhibitor of angiogenesis *in vitro* and exhibited tumor growth inhibition in mice, but N-alkylated levamisole derivatives (N-methyllevamisole and *p*-bromolevamisole) proved more effective than (S)-levamisole hydrochloride, with respect to inhibition of angiogenesis and induction of undifferentiated cluster morphology in human umbilical vein endothelial cells grown in co-culture with normal human dermal fibroblasts[29]. This suggests that investigations of organic acid salts of LMS on inhibition of angiogenesis and tumor growth may be worth carrying out.

Conclusion

In this study, the immune function of CTX-induced immunosuppressed mice was restored and enhanced by organic acid salts of LMS in different degrees, making the spleen and thymus indexes increased as well as the serum IgG and IgM content. The preliminary immunological evaluation indicated the pharmacological effects of LF and LT was better than that of levamisole hydrochloride and the remaining organic acid salts, suggesting some value in clinical application that LF and LT can be administered with a smaller dosage than that of LH. Considering the cost, LT is more acceptable. However, studies on bioavailablity are essential for the full evaluation of organic acid salts of LMS on immune function.

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