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ventricular remodeling in rats“

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Table of contents

Zusammenfassung.....	1
Summary.....	4
1 Introduction	6
1.1 Coronary heart disease and myocardial infarction	6
1.2 Traditional Chinese Medicine	6
1.3 Quality control of traditional Chinese herbs	7
1.3.1 Heavy metals.....	8
1.4 The traditional Chinese herbal formula Si Ni Tang	9
2 Aims and hypothesis.....	11
3 Classification and practice of Si Ni Tang.....	12
3.1 Si Ni Tang and its individual herbs	12
3.1.1 Si Ni Tang	12
3.1.2 Pharmacological activities of SNT	13
3.1.3 <i>Aconitum carmichaelii</i> Debeaux.....	20
3.1.4 <i>Zingiber officinale</i> Roscoe	23
3.1.5 <i>Cinnamomum cassia</i> Presl	26
3.1.6 <i>Glycyrrhiza uralensis</i> Fisch	28
3.2 The influence of traditional processing methods on the chemical profile of <i>A. carmichaelii</i> and interaction of plant constituents	31
3.2.1 Traditional processing of <i>A. carmichaelii</i>	31
3.2.2 Herb-herb interaction - detoxification.....	34
4 Material and methods	36
4.1 Quality control and analysis of the effective components.....	36
4.1.1 Plant material	36
4.1.2 Heavy metal experiments.....	36
4.1.2.1 Reagents	36
4.1.2.2 Preparation of extracts	36
4.1.2.3 Instrumentation and conditions.....	37
4.1.3 HPLC experiments.....	38
4.1.3.1 Reagents	38
4.1.3.2 Preparation of extracts	39
4.1.3.3 Instrumentation and conditions.....	40
4.1.4 NMR experiments.....	43
4.1.4.1 Reagents	43
4.1.4.2 Preparation of extracts	43
4.1.4.3 Instrumentation and conditions.....	45
4.1.5 UV-VIS experiments.....	46
4.1.5.1 Reagents	46

4.1.5.2	Preparation of extracts	46
4.1.5.3	Instrumentation and conditions.....	46
4.2	<i>In vivo</i> experiments.....	47
4.2.1	Drugs and reagents.....	47
4.2.2	Instrumentation	48
4.2.3	Animal models, grouping and medication	48
4.2.4	Echocardiography	49
4.2.5	Blood sampling and tissue collection.....	50
4.2.6	Assay of serum and heart tissue levels by ELISA	50
4.2.7	Statistical analysis.....	51
5	Results	52
5.1	Quality control and analysis of the effective components.....	52
5.1.1	Heavy metal tests	52
5.1.1.1	Calculated intake of arsenic, lead, cadmium and mercury	54
5.1.2	The qualitative fingerprinting of SNT by HPLC.....	55
5.1.3	Identification and quantification of aconitine, hypaconitine and mesaconitine.....	58
5.1.3.1	Optimization of the HPLC chromatographic conditions	58
5.1.3.2	Validation of the HPLC assay.....	59
5.1.3.3	Optimization of the extraction conditions and quantitative analysis	60
5.1.4	Comparison of hypaconitine concentrations in aqueous decoction and methanolic extraction.....	62
5.1.5	Analysis of herbal interactions.....	67
5.1.5.1	Analysis of the interaction between the aconitines with single compounds of <i>Z. officinale</i> , <i>C. cassia</i> and <i>G. uralensis</i>	69
5.1.5.2	Analysis of the interaction between the aconitines with isoliquiritin (a single compound of <i>G. uralensis</i>)	73
5.1.6	Analysis of the formed complex of aconitine and liquiritin.....	76
5.1.6.1	Determination of the stoichiometry by the continuous variations method	80
5.1.6.2	Estimation of the binding constants by the ¹ H-NMR titration method	81
5.1.6.3	Structure identification of the formed complex by 2D-ROESY experiment	87
5.1.6.4	Determination of binding constants by UV-VIS measurements..	90
5.1.7	Conclusion of the phytochemical analysis.....	94
5.2	<i>In vivo</i> experiments.....	95
5.2.1	Effect of treatment with SNT on body weight and heart weight	96
5.2.1.1	Body weight/wet heart weight ratio	98
5.2.2	Echocardiographic analysis.....	100
5.2.3	Molecular biological investigations	103

5.2.3.1 Effects of SNT on collagen markers: collagen I, collagen III and transforming growth factor- β 1	104
5.2.3.2 Effects of SNT on functional index markers: atrial natriuretic peptide and cardiac troponin T.....	117
5.2.3.3 Effects of SNT on inflammatory markers: interleukin-6, interleukin-1 β , tumor necrosis factor- α , high sensitivity C-reactive protein	122
5.2.3.4 Effects of SNT on vascular active markers: endothelin-1 and nitric oxide.....	133
5.2.4 Pathological description of the hearts	138
5.2.5 Histological description of heart and liver	139
5.2.5.1 Heart.....	139
5.2.5.2 Liver	140
6. Discussion.....	142
6.1 Quality control and analysis of the effective components.....	142
6.2 <i>In vivo</i> experiments.....	151
References.....	161
Abbreviations	191
List of figures.....	194
List of tables.....	197
Curriculum Vitae	201
List of publications	202

Zusammenfassung

Hintergrund: Koronare Herzerkrankungen sind verantwortlich für die Hälfte der Todesfälle von kardiovaskulären Erkrankungen und bei 60-70 % der Patienten die Hauptursache für Herzinfarkt (HI). Damit stellen sie eine signifikante Belastung des Gesundheitsbudgets der EU dar. Die traditionelle Chinesische Medizin (TCM) ist ein empirisches Gesundheitssystem, das auf jahrtausendelanger Behandlungserfahrungen in Asien basiert. Die wachsende Beliebtheit der TCM im Westen erfordert die komplexen Mechanismen der Kräutermedizin mit Hilfe von gut etablierten analytischen Methoden der zielorientierten westlichen Medizin zu erforschen.

Ziele: Diese Dissertation beschäftigt sich mit der Evaluierung der chinesischen Kräutermedizin, “Si Ni Tang” (SNT), welche traditionell bei der Behandlung von Herzerkrankungen eingesetzt wird. SNT besteht aus Bestandteilen von vier Pflanzen: *Glycyrrhiza uralensis* Fisch., vorbehandeltes *Aconitum carmichaelii* Debx., *Zingiber officinale* Ross und *Cinnamomum cassia* Prasl.

Das erste Ziel dieser Arbeit war die phytochemische Analyse von SNT und die Evaluierung der Schwermetallegehalte. Durch die Anwesenheit von hoch toxischen Diester-Diterpenoid Alkaloiden (DDAs), Aconitin (AC), Hypaconitin (HA) und Mesaconitin (MA), war der Nachweis der toxikologischen Eigenschaften von SNT zwingend erforderlich.

Das zweite Ziel dieser Arbeit war die Ermittlung des Einflusses von SNT auf das ‚ventrikuläre Remodeling‘ in einem Infarktmodell an der Ratte. Nach 30 Tagen Behandlung wurden echokardiographische Untersuchungen vorgenommen und Herz- und Lebergewebe für pathohistologische Untersuchungen entnommen. Zahlreiche Marker, die im Verlauf des ‚ventrikulären Remodeling‘ eine bedeutende Rolle spielen, wurden im Serum und Herzgewebe der Ratten mittels enzyme-linked immunosorbent assay (ELISA) analysiert.

Resultate: Die Konzentrationen von Blei, Cadmium und Quecksilber der getesteten Chargen lagen unter den von der Europäischen Pharmacopöe für medizinische Pflanzen festgelegten Grenzen.

Mit Hilfe der Hochdruckflüssigkeitschromatographie (HPLC-DAD) wurde ein qualitativer 'Fingerprint' von SNT erstellt. Es konnten Liquiritin, Liquiritigenin, Isoliquiritin, Glyzyrrhizinsäure und Eugenol in SNT identifiziert werden.

Die entwickelte HPLC-DAD Methode erlaubt eine rasche Analyse in nur 23 Minuten pro Durchgang und stellt eine zuverlässige Grundlage für die Quantifizierung der höchst toxischen DDAs in Extrakten von *A. carmichaelii* dar. In dieser Studie wurde HA im Dekokt von nicht vorbehandeltem *A. carmichaelii* nachgewiesen, wobei keine der DDAs im Dekokt von SNT weder mit vorbehandeltem noch mit nicht vorbehandeltem *A. carmichaelii* nachgewiesen werden konnten. Diese Resultate führen zur Annahme, dass SNT als unbedenklich eingestuft werden kann. Gleichzeitig werfen sie die interessante Frage auf, was mit dem HA, das in nicht vorbehandeltem *A. carmichaelii* detektiert wurde, in der Mischung passiert?

Infolge der Untersuchung von Kräuter Interaktionen konnte die Frage beantwortet werden. Liquiritin, Liquiritigenin und Isoliquiritin waren primär verantwortlich für die Interaktion mit HA im Dekokt von SNT mit nicht vorbehandeltem *A. carmichaelii*. Zum ersten Mal konnten Bindungskonstanten für die 1:1 Komplexierung von Liquiritin und Aconitin mit Hilfe von Titrationsexperimenten mit Nuklearer Magnetischer Resonanz bestimmt werden. Diese Entdeckungen bieten neue Möglichkeiten für die Detoxifizierung der DDAs in den *Aconitum* Arten.

In unserer *in vivo* Studie konnten signifikant reduzierte Werte der Kollagen Marker, Kollagen Typ I und Typ III in Serum und Herzgewebe und der Entzündungsmarker, Interleukin-1 β , Tumor Nekrose Faktor- α und high sensitivity C-reactive protein in Serum, verzeichnet werden. Diese Ergebnisse resultieren in einer Abschwächung der Fibrosierung und bemerkenswerte anti-entzündliche Effekte, die mit einer Prävention von ‚ventrikulären Remodeling‘ assoziiert sind. Zusätzlich konnte SNT die Serumkonzentration des vaskular aktiven Markers, Stickoxid, erhöhen. Diese Resultate sind übereinstimmend mit der Verbesserung der linksventrikulären Funktion und der Prävention von kardialer Dilatation, die in echokardiographischen und pathohistologischen Untersuchungen ermittelt wurden. Die Serum Werte der folgenden fünf Marker, transforming growth factor- β 1, atrial natriuretic peptide, cardiac troponin T, Interleukin-6 und Endothelin-1, unterschieden sich nicht signifikant zwischen den mit SNT behandelten Gruppen und der Modellgruppe.

Schlussfolgerung: Unsere Ergebnisse zeigen, dass die Verabreichung von SNT an Ratten nach einem induzierten HI positive Effekte aufweist und diese rechtfertigen daher den traditionellen Gebrauch von SNT in China. Unsere Studie bietet eine effiziente Methode zum Arzneimittel Screening, um den Einfluss von Arzneien auf ‚ventrikuläres Remodeling‘ nach einem HI zu ermitteln und leistet daher einen wichtigen Beitrag zur Herz-Kreislauf-Forschung.

Summary

Background: Coronary heart disease is the main cause for myocardial infarction (MI) in 60-70 % of patients suffering from cardiovascular disease and accounts for half of their deaths. Coronary heart disease creates a significant impact on the health-care budgets of the EU economy. Traditional Chinese Medicine (TCM) is an empirical healthcare system based on several millenia of human treatment in Asia. Due to TCM's growing popularity it is necessary to bridge the gap to Western medicine and to explain the herbal medicine's complex mechanism of action using the well- established analytical methods developed for target-oriented Western medicine.

Aims: Our overall aim was to evaluate the Chinese medicinal formula, "Si Ni Tang" (SNT) which is traditionally used for the treatment of cardiac diseases. SNT consists of parts of the four plants *Glycyrrhiza uralensis* Fisch., pretreated *Aconitum carmichaelii* Debx., *Zingiber officinale* Ross. and *Cinnamomum cassia* Prasl.

The first objective of this thesis was the phytochemical analysis of SNT and the determination of the presence of heavy metals. The analysis of the toxicological characteristics of SNT was imperative due to the highly toxic diester diterpene alkaloids (DDAs), aconitine (AC), hypaconitine (HA) and mesaconitine (MA) which are present in raw *A. carmichaelii* roots.

The second objective of this thesis was to examine the effects of SNT on post-infarction ventricular remodeling in rats. In order to test the effects of SNT, echocardiography was performed after 30 days of treatment and heart and liver tissues were removed for pathohistological examinations. In addition, various markers known to be involved in the process of cardiovascular remodeling were analyzed in the serum and heart tissue of rats, using enzyme-linked immunosorbent assay (ELISA).

Results: Concentrations of lead, cadmium and mercury in the batches that were tested did not exceed the limits recommended for medicinal plants by the European Pharmacopoeia.

The qualitative fingerprinting analysis by high-performance liquid chromatography diode array detector (HPLC-DAD) method revealed liquiritin, liquiritigenin, isoliquiritin, glycyrrhizic acid and eugenol as components in the SNT.

The HPLC-DAD method, which allowed a quick analysis time (23 min per passage), provided a reliable basis for the quantification of the highly toxic DDAs in the extracts of *A. carmichaelii*. In this study, HA was found in the decoction of unprocessed *A. carmichaelii* whereas none of the DDAs were detectable in the SNT decoction containing either processed or unprocessed *A. carmichaelii*. These results suggested that SNT is safe to use, but they also provoked the interesting question of what happened to the HA detected in unprocessed *A. carmichaelii* when it was combined in the whole formula?

The analysis of the herbal interaction answered the question: liquiritin, isoliquiritin and liquiritigenin were identified to be primarily responsible for interacting with HA in the SNT decoction containing unprocessed *A. carmichaelii*. For the first time binding constants were determined for the complexation of 1:1 stoichiometry between liquiritin and aconitine by carrying out titration experiments with nuclear magnetic resonance. These discoveries offer new possibilities for the detoxification of the DDAs in the *Aconitum* species.

According to this study, SNT significantly reduced the serum and cardiac tissue levels of collagen makers, collagen type I and type III, and the serum levels of inflammatory markers, interleukin-1 β , tumor necrosis factor- α and high sensitivity C-reactive protein. The result of this was an attenuation of fibrosis and various marked anti-inflammatory effects, which are associated with the prevention of cardiac remodeling and, as a consequence, heart failure. In addition, SNT raised the serum level of the vascular active marker nitric oxide. These observations correspond to the improvement in the left ventricular function and to the prevention of cardiac dilatation, both of which could be seen in the echocardiography and pathological examinations. The levels of the five markers, transforming growth factor- β 1, atrial natriuretic peptide, cardiac troponin T, interleukin-6 and endothelin-1, were not found to be significantly different in the SNT treated groups to those of the model group.

Conclusions: Our results indicate the beneficial effects of the SNT administration to rats post-MI and therefore provide justification for its traditional use in China. Our study represents an important contribution to cardiovascular research by providing an efficient method of drug screening which can be used to determine the drug's influence on cardiac remodeling post-MI.

1 Introduction

1.1 Coronary heart disease and myocardial infarction

Coronary heart disease (CHD) is the main etiology for myocardial infarction (MI) in 60–70 % of patients ^{1,2} and CHD accounts for about half of the total deaths from cardiovascular disease (CVD) ³. Due to a total of 17 million deaths p.a. globally in 2002 ⁴ and over 2 million deaths p.a. in the European Union (EU) in 2008 ⁵, CVD is one of the main health conditions creating a significant impact on the health-care budgets of the EU economy with an estimated cost of € 192 billion a year ⁵.

In the World Health Organization (WHO) Burden of Disease project in 2002 the DALY (Disability Adjusted Life Year) was used as the indicating factor. This signifies ‘an aggregate of years of life lost due to premature death and years of healthy life lost due to disability’ ⁶. In the EU, nearly 5 million DALYs (8 % of total DALYs lost) are lost each year due to CHD ⁵. In Europe the numbers are even more tragic, with over 15 million DALY’s lost each year because of CHD. In the Ukraine, Russia and Belarus the age-standardized rates of DALYs lost due to CHD are at least three times higher than in Spain, France and Italy ⁵.

At the same time the risk of myocardial infarction is, in a worrying development, steadily increasing and is a leading cause of death. These data and the fact of a continuous rise in incidence, indicate a necessity for novel therapeutic agents to support conventional treatments in post-infarction ventricular remodeling. In addition, new pharmaceutical approaches aim to treat the patient post-MI and to improve their quality of life.

1.2 Traditional Chinese Medicine

Traditional Chinese Medicine (TCM) is an empirical healthcare system based on human application in Asia for several millennia. The first written records originated in the period of the Western Zhou Dynasty 1100-770 B.C. and the various aspects include Chinese material medica (also known as Chinese herbal medicine), acupuncture, moxibustion (burnt mugwort therapy), tuina (massage), qigong (movement and breathing exercise), tai chi (slow movements, defense training) and others ⁷. One of the first collections of TCM is the ‘Huangdi’s International

Classic' which was probably finished during the Warring States in 475-211 B.C. and still is a must-read for all TCM practitioners. Prior to the implementation of modern medicine by English missionaries in the early nineteenth century, TCM provides the only medical treatment in China. The development of TCM is based on empirical testing and refinement over thousands of years ⁸. Reports indicate that over 10 % of the U.S. population used herbal medicines in 1999 ⁹. About 70-80 % of the world population uses alternative medicine, mainly herbal, according to a WHO study from 2002 to 2005 ¹⁰. The use of TCM drugs has increased steadily in recent decades in the western countries in the field of alternative medicine ¹¹⁻¹³. TCM has found its way into the medical care in Europe too. TCM products around US \$ 2 billion were imported in European countries in 2010 showing an increase of 10 % per year ⁸. In the United Kingdom, about one in ten people used TCM in 2010, however in Switzerland and the Scandinavian countries the number of TCM-consumers lay between 30 and 50 % ⁸.

This increase in demand necessitates more advanced evidence, based on medical evaluation methods according to scientifically approved and accepted standards. In this way the most effective and safe use of TCMs can be guaranteed ¹².

1.3 Quality control of traditional Chinese herbs

“Despite its existence and continued use over many centuries, and its popularity and extensive use during the last decade, traditional medicine has not been officially recognized in most countries. (...) The quantity and quality of the safety and efficacy data on traditional medicine are far from sufficient to meet the criteria needed to support its use world-wide. The reasons for the lack of research data are due not only to health care policies, but also to a lack of adequate or accepted research methodology for evaluating traditional medicine” as quoted in “General Guidelines for Methodologies on Research and Evaluation of Traditional Medicines” ¹⁴.

A great challenge in quality control (QC) is the authentication and identification of herbs. Mistaken identity of the plants followed by toxicopathy often occurred in the past and must be avoided by the experts who harvest the herbs, and an effective QC must be efficiently established. For the identification of the single herbs, in general, one or two markers, mainly bioactive components of the herb, are defined and used for the evaluation of the quality and

authenticity. However, this kind of analysis is abstract and disregards the fact that the therapeutic effects of a herb are due to ‘synergistically working’ multiple constituents¹⁵.

Furthermore, the chemical constituents in component herbs vary depending on harvest seasons, plant origins, drying factors, and others. Also, the characteristic preparations of TCM, the extraction (either of single herbs or as collections of herbs in herbal mixtures) with boiling water during the decoction process, makes QC of TCMs more difficult than that of western drugs¹⁵. In addition, all the main compounds of the single drugs have to be considered in the QC of herbal mixtures¹⁶ and the herbal interaction contributes to the complexity of QC of TCMs.

Liang et al. reports in his review on ‘qualitative and quantitative analysis in QC of TCMs’ that the most direct and important method for QC of TCMs is the quantitative analysis of active components. He also mentions the necessity for further research on the chemical constituents and the biological screening¹⁷. In the case of authentication and identification of herbal products the analysis of the chromatographic fingerprints should be the method selected even if the concentration of bioactive compounds is not identical in the various samples. The chromatographic fingerprints should present both the ‘sameness’ and the ‘differences’ between the samples¹⁵.

These indicate that the QC is one of the main difficulties faced in the application and development of TCM’s¹⁷ and that the need for evidence-based scientific QC methodology, which uncovers adulteration, misidentification, the variability in the amount of active ingredients and an improper processing and preparation¹⁸, is urgent.

1.3.1 Heavy metals

Heavy metals are naturally present in TCM herbs. Their occurrence, however, has gradually risen with the increase of industrialization. Heavy metal poisonings associated with the intake of traditional medicinal plants were reported and discussed in India¹⁹⁻²¹, China²²⁻²⁴, Brazil²⁵, Argentina²⁶, Mexico²⁷, the U.S.²¹, Bulgaria²⁸, Serbia²⁹ and Poland³⁰. Contamination of raw herbal products can occur due to many complex factors such as cultivation, processing or time of harvesting. The level and duration of contaminant exposure due to metal mining, topography, geographical origin and storage also play a role^{25,28}. High levels of toxic metals can be found

when agricultural expedients are used, for example fertilizers with cadmium content, mercury or lead based pesticides³¹ or in medicinal preparations where heavy metals are used as active ingredients, as in the case of lead and mercury present in some Chinese, Mexican and Indian medicines^{21,32,33}.

Cadmium, mercury, lead and arsenic are among the most abundant heavy metals in these plants²³. The European Pharmacopoeia 7th Edition 2011 (volumes 7.0-7.2) points out that analyzing the heavy metal contents is an important factor in QC³⁴. The upper limit determined by the EU-Commission is for lead 5 mg/kg, for cadmium 1 mg/kg and for mercury 0.1 mg/kg. The limit for arsenic has not yet been defined.

1.4 The traditional Chinese herbal formula Si Ni Tang

Compared to target-oriented Western medicine, TCM is based on an integrated and synergistic approach, focusing on the balance of Yin and Yang, and consequently the restoration of body energy and homeostasis³⁵. This ancient medicine differs in matter, methodology and philosophy to modern medicine and therefore brings some misconceptions about its full implication⁸. For well-specified diseases without any complications single herbs are usually prescribed^{36,37}. By contrast, for the treatment of complicated disease and chronic diseases formulas containing several herbs are often required to obtain maximum benefits and minimize possible side effects^{36,37}. Due to TCM's growing popularity it is necessary to build a bridge between TCM and Western medicine and to endeavor to explain the complex mechanism of action of the herbal medicine using the well-established scientific methods developed for target-oriented Western medicine.

Earlier studies have already provided evidence to support the use of TCM as therapeutic remedies for myocardial infarction^{12,38-45}. As a result, the treatment of cardiac disease with TCM is now attracting more interest in western countries. In this study we have evaluated a representative Chinese medicinal formula, "Si Ni Tang" (SNT). It has a tradition beginning between 150 and 219 A.D. for the treatment of CVD and has been officially recorded in Chinese pharmacopoeia, in the 2010 edition⁴⁶.

SNT consists of parts of three plants, the radix of *Aconitum carmichaelii* Debx. (aconite, Zhi Fu Zi), the rhizoma of *Zingiber officinale* Ross. (ginger, Gan Jiang) and the radix of *Glycyrrhiza uralensis* Fisch. (licorice, Zhi Gan Cao), combined following a traditional formula from ancient times. It is used in clinics in China to improve blood circulation, relieve blood stasis, for treating myocardial damage and cardiac exhaustion. We modified the herbal mixture SNT for our studies by adding the bark of *Cinnamomum cassia* Prasl. (cinnamon, Rou Gui). According to TCM theory Rou Gui, one of the most internal-warming herbs, dispels cold, relieves pain and opens channels and vessels. In the book ‘Chinese Medical Herbology and Pharmacology’ it is reported that an intravenous administration of Rou Gui reduces blood pressure, decreases the heart rate, vessel resistance and peripheral vasodilatation within 3 to 5 min. after injection³⁷. Our decision to modify SNT with Rou Gui was based on its cardiovascular, analgesic and antibiotic properties, the protection it provides for the gastrointestinal system and the stimulation of the immune system. In this way we could increase the therapeutic effect, decrease any potential side effects and harmonize the mixture.

2 Aims and hypothesis

The first objective of this thesis is the phytochemical investigation of Si Ni Tang (SNT), a Chinese herbal formula consisting of the radix of *A. carmichaelii*, the rhizoma of *Z. officinale*, the cortex of *C. cassia* and the radix of *G. uralensis*. The phytochemical analysis covers the evaluation of the toxicological characteristics of SNT, possible herbal interaction and heavy metal contamination. In order to do this, standardized preparation procedures, methods and protocols have been developed for the use of high-performance liquid chromatography diode array detector (HPLC-DAD), nuclear magnetic resonance (NMR), microwave reaction system and inductively coupled plasma-optical emission spectroscopy (ICP-OES). For the phytochemical analysis, decoctions of individual herbs, various combinations and whole SNT mixtures are prepared and chemically analyzed to obtain information about possible herbal interactions. The same procedure and the same concentrations are used in all experiments to make the results comparable. It is in everybody's interest to keep the analysis as practice-orientated as possible. This analysis provides a guideline for the safe use of SNT.

The second objective is to investigate the effects of SNT on post-infarction ventricular remodeling in rats. In order to prove the effects of SNT various markers known to be involved in the process of cardiovascular remodeling have been selected and analyzed in serum and heart tissue of rats:

Collagen markers (measured in serum and heart tissue): collagen I, collagen III and transforming growth factor- β 1

Functional index markers (serum): atrial natriuretic peptide and cardiac troponin T

Inflammatory markers (serum): interleukin-6, interleukin-1 β (serum and tissue), tumor necrosis factor- α and high sensitivity C-reactive protein

Vascular active markers (serum): endothelin-1 and nitric oxide

These observations reveal if and how SNT acts in the treatment of cardiovascular disease. The main focus is to examine if there is a significant improvement in post-myocardial infarction remodeling. This study has been funded as a Joint Research Project by the Austrian Ministry of Science and Research, the Austrian Ministry of Health and the Ministry of Science and Technology of the People's Republic of China, who are interested in establishing the effective and safe use of TCM in Europe.

3 Classification and practice of Si Ni Tang

3.1 *Si Ni Tang and its individual herbs*

3.1.1 Si Ni Tang

Si Ni Tang, a traditional Chinese herbal formula, is used as a decoction to ‘warm’ the inner body and dispel ‘colds’. It restores the influence of the ‘Yang Qi’ and is described as a ‘decoction for treating yang exhaustion’. SNT is well-known to be very effective in treating patients with shock, heart failure, severe watery diarrhea and poor circulation of the extremities ⁴⁷. It is also used for the prevention of chronic heart failure post-myocardial infarction ⁴⁸. In figure 1 you can see the components of SNT and the table 1 presents an overview of the single compounds of SNT.

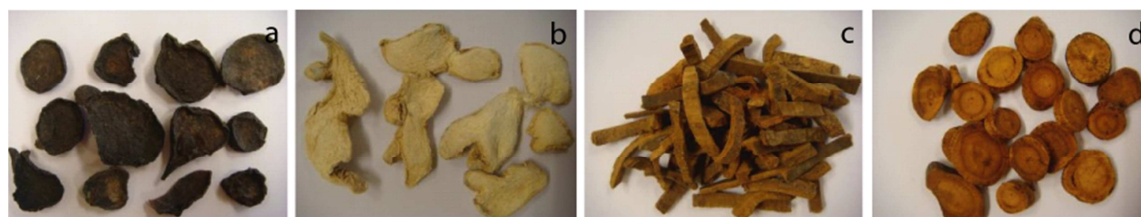


Figure 1. Components of SNT (image: K. Peter), (a) Zhi Fu Zi (b) Gan Jiang (c) Rou Gui (d) Zhi Gan Cao

Table 1. SNT is composed of parts of three plants and modified by the addition of the bark of *C. cassia*

Chinese	Latin	Used part of plant	Ratio
Zhi Fu Zi	<i>Aconitum carmichaelii</i>	Radix (lateral root tuber)	6
Gan Jiang	<i>Zingiber officinale</i>	Rhizoma	3
Rou Gui	<i>Cinnamomum cassia</i>	Cortex	1
Zhi Gan Cao	<i>Glycyrrhiza uralensis</i>	Radix	8

3.1.2 Pharmacological activities of SNT

SNT is a very common and popular herbal formula in China and Japan (Japanese name: Shigyaku-to). These modern pharmacological studies and clinical trials of SNT, focusing on cardiovascular disease and its mechanisms, legitimate its traditional use.

Wu et al. studied in 1994 and 1995 the mechanisms of the protective effect of SNT on ischemic myocardium in mice. They observed a significant suppression of lipid peroxidation of myocardial homogenate due to SNT's effective scavenging for oxygen free radicals⁴⁹. SNT can significantly reduce the content of malondialdehyde in ischemic myocardium. Individual ingredients, *Radix Aconitum* and *Radix Glycyrrhizae*, also show this effect but less effectively than the entire SNT complex⁵⁰. Furthermore, there were indications of an increase of nutritional blood flow and a rise in the activity of superoxide dismutase in ischemic myocardium⁵¹.

In addition, Wu and his team studied the effect of SNT on myocardial endothelin (MET) in myocardial ischemia induced in rats and observed that SNT could significantly lower MET. In immunohistochemical examination they found out that this effect may be related to an inhibition of MET-1 gene expression and protein synthesis by SNT⁵².

Another study of Wu et al. in 2001 reported on the ability of SNT to improve reperfusion injury of thrombolytic therapy in patients with acute myocardial infarction. They observed that the period of acute ST segment, total burden of myocardial infarction, QRS score, QT dispersion and the occurrence of reperfusion arrhythmia in electrocardiography all showed lower levels in SNT-treated patients, than in SNT-untreated patients⁵³.

In 1997, Su et al. conducted a study on hemorheology following percutaneous transluminal coronary angioplasty (PTCA). Hemorheologic variables of forty-six patients were measured before and after SNT treatment. They observed a significant reduction in whole blood viscosity and red cell aggregation in SNT patients in comparison to the patients with only PTCA and without SNT treatment, where no hemorheologic changes were recorded⁵⁴.

Liu et al. investigated the effect of SNT in the prevention of vascular restenosis and the protection against oxidative stress after rabbit iliac artery balloon injury. The vascular lumen and intima thickness remained basically normal in the SNT group (balloon injury and SNT administration), whereas microscopic examination revealed narrowed vascular lumen, thickened intima and numerous arteriosclerotic plaques in the model group (balloon injury, no SNT administration) when compared to the control group (no subjection to balloon injury, no SNT administration). The comparison also showed that the levels of total cholesterol, low density lipoprotein cholesterol, and triglyceride were lower and high density lipoprotein cholesterol levels higher in the SNT group compared to the model group. The serum malondialdehyde level was lower in the control group and the SNT group showed a lower level than the model group. The serum superoxide dismutase activity was higher in the control group, but a higher level was determined in the SNT group than that of the model group⁵⁵.

Zhao et al. studied the role of the superoxide dismutase (SOD) in Adriamycin (ADR)-induced heart failure and the protective effects of SNT. Three weeks after injection of ADR in the caudal vein, the cardiac function, the activity and the mRNA expression of Cu-Zn SOD and Mn SOD had significantly increased and the level of malondialdehyde had decreased in the SNT group (induction of heart failure and administration of 3.75 g/kg/d), which showed no statistic difference in comparison with the results of the control group (no induction of heart failure, no administration of SNT)⁵⁶. Furthermore, mitochondrial swelling could be observed and the levels of Na⁺-K⁺ ATPase (sodium-potassium adenosine triphosphatase) and Ca²⁺ ATPase (calcium adenosine triphosphatase) were measured. They found that Na⁺-K⁺ ATPase and Ca²⁺ ATPase had increased significantly and that the degree of mitochondria swelling was lower in rats treated with SNT than in the model group (induction of heart failure, no administration of SNT). The authors suggest the presence of oxidative stress in mitochondria of myocardium in ADR-induced heart failure rats and the positive effect of SNT on it⁵⁷.

In 2005, Liu et al. examined whether SNT could induce a delay in the preconditioning-like effect on the rat heart and its possible mechanism. The left anterior descending coronary artery was occluded for one hour and reperfused. In comparison to the model group (occlusion and reperfusion, no SNT administration), the SNT group (occlusion and reperfusion, pretreatment

with SNT (5 g/kg/d for three days prior to the occlusion) showed the following results: the size of the myocardial infarct and the content of malondialdehyde in myocardial tissue were significantly lower, the activity of superoxide dismutase (SOD) and the mRNA expression of MnSOD were higher. The translocation of NF- κ B (nuclear factor 'kappa-light-chain-enhancer' of activated B-cells) was identified and moreover the expression of NF- κ B had significantly increased. These data suggest that the SNT induces a delay in the precondition-like effect in the rat heart and that the activation of NF- κ B may play an important role in this process ⁵⁸.

Qin et al. published a paper in 2004 on the clinical action of SNT on angina pectoris (AP), and they compared the effect of SNT or isosorbide dinitrate (ID) on the life quality of the AP patients, and the effects using a combination of both western and traditional medicine. It was reported that both SNT and ID relaxed AP, led to improved electrocardiography results and improved the rate of life quality, although SNT showed better results regarding the life quality than ID. However, the best clinical effect was achieved by combining SNT and ID ⁵⁹.

Similar results had been drawn in 2000 by Su et al., when they studied the influence of SNT on the life quality of patients after percutaneous transluminal coronary angioplasty (PTCA). SNT could significantly improve the levels of physical symptoms particularly in relieving palpitation and dyspnea, a general sense of well-being and a lesser degree of depression compared to the patients with only PTCA and no SNT administration ⁶⁰.

The next study deals with the observation of the clinical effect of SNT on stenocardia in coronary heart disease with deficiency of yang or cold syndrome. SNT patients showed similar effects to those of isosorbide dinitrate (ID) patients, an improvement in the clinical symptoms and electrocardiography, a decrease in the frequency of stenocardia and a reduced dose of nitroglycerin, but SNT showed better effects than ID on the reduction of myocardial oxygen consumption and the improvement of cardiac function ⁶¹.

The effects of SNT on microcirculatory disturbances induced by endotoxin in rat mesentery were investigated by Zhang et al. in 1999. The authors suggest that SNT has protective properties which improve the microcirculatory disturbances by preventing of a decline of the mean arterial

pressure, controlling the reduced velocity and suppressing the leukocyte extravasation across veins⁶².

A positive effect of SNT on post-percutaneous transluminal coronary angioplasty ischemia-reperfusion (PTCA) injury was observed by Wu et al. The patients of the SNT group received 25 mL of SNT decoction daily three days prior to and three days after the operation. In the SNT group, there was relief from the deficiency of superoxide dismutase activity, and the nitric oxide content and level of malondialdehyde level were significantly higher than in the patients only with PTCA and no SNT administration⁶³.

In a study in 2008, Dietl et al. examined the fact that SNT has a significant positive effect on cardiac function after myocardial infarction (MI) and that this influence might be due to its anti-apoptotic effects. The results show a reduction in left ventricle/body weight-ratio, an attenuation of end-diastolic and –systolic dilation and a significantly higher fractional shortening in SNT rats (MI induction and SNT administration) as compared to the model group (MI induction, no SNT administration). Furthermore, the apoptosis was measured using an in situ cell death detection kit where a significant reduction of apoptotic cells was found in comparison to the model group⁴⁸.

Similar results were reported by Liu et al. They observed that the delay in the effect of preconditioning induced by SNT reduced myocardial cell apoptosis. The rats in the SNT group were pretreated with SNT (5 g/kg/d for three days prior to the occlusion followed by reperfusion of the left anterior descending artery (LAD)). A significant decrease in the cytochrome C released by mitochondria, a decrease of the activity of Caspase-3 and an increase of the expression of Bcl-xL protein in the SNT group as opposed to the model group (occlusion and reperfusion, no SNT administration) all suggest that the positive influence of SNT on cell apoptosis is related to the inhibition of the mitochondria signal pathway of apoptosis^{64,65}.

In 2008 the same group around Liu et al. performed a study on the effects of protein kinase C (PKC) activation and Smac (second mitochondria-derived activator of caspase) release and whether SNT had an influence on these markers to improve the inhibition of myocardial cell apoptosis. They again observed a decrease in myocardial cell apoptosis. In addition, PKC was

translocated and its expression raised. Both, Smac release and the activity of Caspase-3 were reduced. The protective effect of SNT on myocardial cell might be attributable to PKC activation and mitochondria protection, which in turn inhibit Smac release and inhibit apoptosis signal transduction⁶⁶.

In 2009 Zhao et al. did research on the protective effects of SNT on Adriamycin-induced heart failure in rats. In the SNT group cell apoptosis was decreased, protein expression of Bcl-xL and Bcl-xL/Bcl-xS mRNA increased in ratio and Bid protein expression decreased in comparison to the model group (induction of heart failure, no SNT administration). The authors suggest that Bcl-xL may play a considerable role in heart failure and that the mechanism of SNT on the protection of cardiocyte might be attributable to the apoptosis correlation factors, Bcl-xL and Bid⁶⁷.

A very complex study was published recently by Tan et al., where a urinary metabonomic method was developed based on nuclear magnetic resonance and high performance liquid chromatography-mass spectrometry in order to characterize myocardial infarction (MI)-related metabolic profiles and define the influence of SNT on these. The echocardiographic results indicate a significant improvement in the left ventricle systolic function, an ejection fraction and a fractional shortening in rats treated with SNT (ligation of the artery, SNT administration) when compared to the MI (ligation of the artery, no SNT administration) and the Sham operated group (operation without ligation, no SNT administration). Furthermore, the examination of heart tissue samples show that SNT could prevent the development of cardiac dilatation post-MI, in contrast to the MI group where an increased chamber dilatation occurred. In addition, it was reported that the infarct areas of the SNT-treat group were significantly smaller in comparison to the MI group. The metabolite profiling in this study results in a down-regulation of lactate, succinate, and L-alanine and an up-regulation of citrate, oxalosuccinate, 2-oxoglutarate, and L-tryptophan in the SNT-treated group compared to the MI group. The fact is that myocardial ischemia and oxygen deficiency implicate a high rate of glycolysis, lactate accumulation and inhibition of citrate cycle and SNT has the ability to intervene in these cycles. In addition the echocardiographic results suggest that SNT has extensive effects on MI due to the regeneration of the unbalanced myocardial energy metabolism³⁵.

Another study by Tan and his colleagues published recently deals with the determination of 21 potential biomarkers involved in the pathophysiological change of myocardial tissue and comparison drawn between an MI group (ligation of the artery, no SNT administration), a Sham operated group (operation without ligation, no SNT administration) and a SNT-treated group (ligation and SNT administration). SNT was able to significantly reverse the level of eighteen biomarkers at different degrees in the serum of MI rats; for example LPC, L-valine, L-tryptophan, L-isoleucine, Palmitoyl- L -carnitine, creatine and arachidonic acid. These results imply that the unique effects of SNT in the treatment of MI are due to partially restoring the imbalance of sphingolipid-, L-tryptophan-, L-arginine-, L-proline-, and branched-chain amino acid (BCAA)-metabolism, phospholipid catabolism, and fatty acid transportation and metabolism⁶⁸.

Overview of SNT's pharmacological activities

To summarize, these studies justify the traditional use of SNT in the treatment of cardiovascular diseases. Different pathways were observed by several investigators and extensive properties of SNT were documented:

- improvement of blood flow, reduction of whole blood viscosity and red cell aggregation
- improvement of electrocardiography: reduction of acute ST segment, total burden of myocardial infarction, QRS score, QT dispersion and the occurrence of reperfusion arrhythmia
- improvement of echocardiography: reduction of left ventricle/body weight-ratio, attenuation of end-diastolic and systolic dilation, improvement in left ventricle systolic function and ejection fraction, increased fractional shortening
- reduction of infarct areas
- reduction of myocardial consumption
- reduction of malondialdehyde
- increase of the activity of superoxide dismutase (SOD)
- increase of mRNA expression of Cu-Zn SOD and Mn SOD
- translocation of NF- κ B and increased expression of NF- κ B
- increase of Na⁺-K⁺ ATPase and Ca²⁺ ATPase

- reduction of myocardial endothelin (MET) due to the inhibition of MET-1-gene expression
- reduction of total cholesterol, low density lipoprotein and triglyceride, increase of high density lipoprotein level
- improvement in life quality of patients
- improvement of physical symptoms, particularly in relieving palpitations and dyspnea, sense of well-being and lesser degree of depression
- anti-apoptotic effects due to inhibition of apoptosis signal transduction: translocation of protein kinase C and elevation of expression, inhibition of Smac release, decrease of the release of cytochrome C from mitochondria, reduction of the activity of Caspase-3, increase of Bcl-xL protein expression, reduction of Bid protein expression
- regeneration of the unbalanced myocardial energy metabolism post-myocardial infarction
- restoration of the imbalance of sphingolipid-, L-tryptophan-, L-arginine-, L-proline-, and branched-chain amino acid (BCAA)-metabolism, phospholipid catabolism, and fatty acid transportation and metabolism
- shortening of colon transmission time in functional constipation patients ²

It seems that the SNT acts simultaneously on several targets involved in the protection of the heart and vascular system. To our knowledge, the effect of SNT on ventricular remodeling post-MI has not yet been investigated. Finding out if and how SNT takes part in the complex mechanisms of ventricular remodeling is a matter of great interest.

3.1.3 *Aconitum carmichaelii* Debeaux

Family: Ranunculaceae

Chinese Name: (Zhi) Fu Zi

English name: (pretreated) aconite

Used part: root and tuber

Aconitum is well-known worldwide as a source of both arrow poisons and medicine. In figure 2 the plant parts of *A. carmichaelii* are shown. The diester diterpene alkaloids (DDAs), aconitine, hypaconitine and mesaconitine (see figure 3) are responsible for its high cardiotoxicity and neurotoxicity⁶⁹. Aconitine, the most toxic of these alkaloids, is reported to be an activator of the voltage-dependent Na⁺ channel. Aconitine shows a positive inotropic effect due to prolonging the effect of sodium influx during the action potential⁷⁰. Further studies indicate various interactions with the Na⁺ channel depending on the chemical structure of the alkaloids⁷¹.



Figure 2. Plant parts of *A. carmichaelii* (a) Flower and leaves of *A. carmichaelii* (image: http://en.wikipedia.org/wiki/Aconitum_carmichaelii), (b) Fu Zi, dried aconite roots and tubers (image: K. Peter) (c) Zhi Fu Zi, pretreated and dried aconite roots and tubers (image: K. Peter)

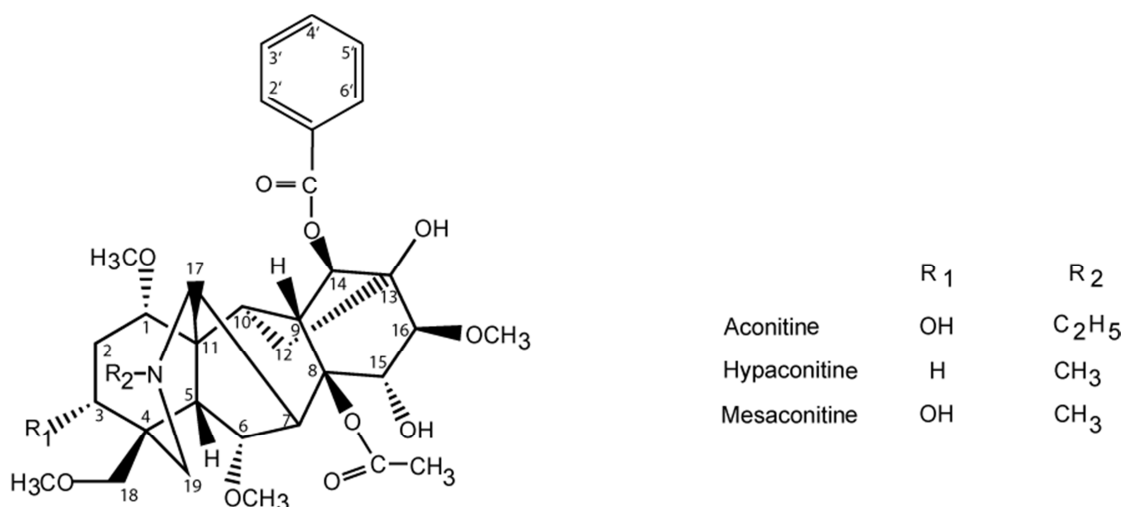


Figure 3. Chemical structure of aconitine, hyaconitine and mesaconitine

According to the theory of TCM, Fu Zi symbolizes to the heart, kidney and spleen meridians. Fu Zi restores depleted yang, tonifies yang and augments fire, warms channels, disperses cold and dampness and relieves pain³⁷. The roots and tubers of *Aconitum* are widely employed in TCM in the treatment of pain⁷², neuronal disorders⁷³, and for cardiotoxic treatment⁷⁴. Furthermore, Singhuber et al. reported a common application of *A. carmichaelii* for various diseases such as collapse, syncope, rheumatic fever, painful joints, gastroenteritis, diarrhea, edema, bronchial asthma, various tumors, and some endocrinal disorders such as irregular menstruation⁷⁵.

Cardiovascular activity

In the book 'Chinese Medical Herbology and Pharmacology' it is reported that the administration of Fu Zi is associated with positive inotropic and chronotropic effects. An intravenous injection of Fu Zi in animals indicated increased cardiac contractility, dilated blood vessels, and increased cardiac output. Fu Zi also showed beneficial effects when treating shock in cats and rats, as it increases blood pressure and cardiac contraction³⁷.

Several studies of Chinese and Japanese scholars confirmed the cardiac effects of Fu Zi⁷⁶. A variety of cardioactive components of *A. carmichaelii* were isolated such as

- DL-demethylcoclaurine⁷⁷, suitable for the clinical treatment for chronic arrhythmia and low cardiac output syndrome⁷⁸;

- coryneine chloride (a dopamine derivate with α -adrenergic effects)⁷⁰, which can improve blood pressure⁷⁹;
- higenamine, a β -agonist with positive chronotropic and inotropic effects⁸⁰ and
- uracil and fuzinoside isolated from Han et al. and Xu et al.^{81,82}.

Liu and his co-workers isolated five cardioactive C₁₉-diterpenoid alkaloids: N-deethylaconine, beiwutinine, hypaconine, mesaconine, and 15 α -hydroxyneoline. Hypaconine, mesaconine and beiwutinine have optimal cardiac actions whereas mesaconine shows protective effects, including an improved inotropic effect and left ventricular diastolic function on myocardial ischemia-reperfusion injury in rats⁷⁶. Singhuber et al. illustrated in her review a variety of aconitine alkaloids and their pharmacological effects⁷⁵.

3.1.4 *Zingiber officinale* Roscoe

Family: Zingiberaceae

Chinese name: Shen/Gan Jiang

English name: fresh/dried ginger

Used part: rhizome

Ginger is not only popular worldwide as a condiment and flavoring agent, but is also used globally as a remedy for nausea and spasms further as a warming aid in the case of colds and chills⁸³. In figure 4 the plant parts of *Z. officinale* are shown. In China ginger has been used for at least 2500 years as an antiemeticum, a digestive remedy, and for treating bleeding disorders, rheumatism, baldness, toothache, snakebite and respiratory conditions⁸⁴. According to TCM theory, Gan Jiang is symbolized to the spleen, kidney, stomach, heart and lung meridians. Gan Jiang is known as a pungent, dry, warming, yang herb and is used for ailments triggered by cold, damp weather⁸³.



Figure 4. Plant parts of *Z. officinale* (a) chromolithography plate of ginger from Köhler's Medicinal Plants (image: <http://de.wikipedia.org/wiki/Ingwer>), (b) whole plant (image: K. Peter) (c) Shen Jiang, fresh Rhizoma Zingiberis (image: K. Peter) (d) Gan Jiang, dried Rhizoma Zingiberis (image: K. Peter)

The major active chemical, non-volatile constituents of ginger are the gingerols with varying chain lengths and their dehydrated forms: the shogaols (see figure 5). Gingerols, a homologous series of phenols, are degraded during thermal processing into corresponding shogaols⁸⁵. They are responsible for the pungency of ginger – gingerol in fresh ginger and shogaol in dry ginger⁸⁶.

The concentrations of the numerous constituents vary depending on the place of origin and whether the rhizomes are fresh, dried or processed ⁸⁷.

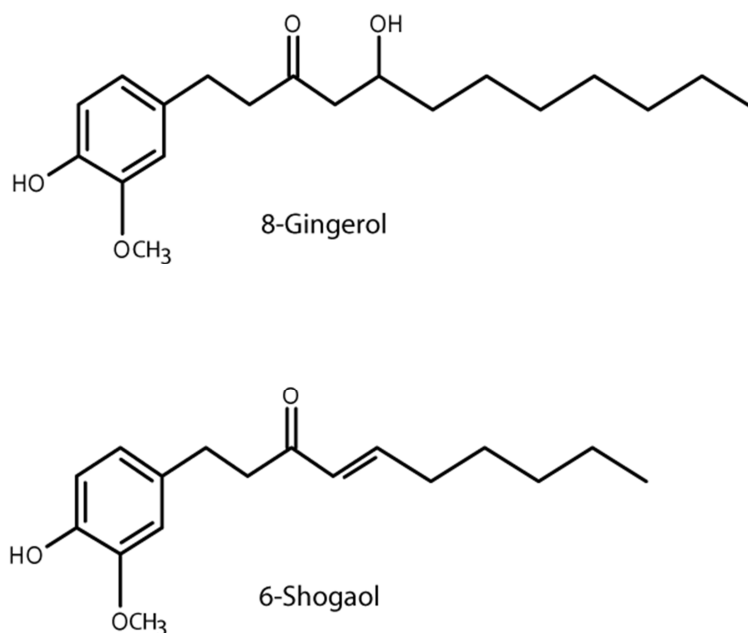


Figure 5. Chemical structure of 8-Gingerol and 6-Shogaol

In recent years, experimental studies have provided information on the various pharmacological properties of ginger:

- anti-inflammatory ⁸⁸⁻⁹²,
- neuroprotective ⁹³⁻⁹⁷,
- chemopreventive ⁹⁸⁻¹⁰³,
- anti-cancer ¹⁰⁴⁻¹⁰⁷,
- anti-metastatic ¹⁰⁸,
- anti-microbial ¹⁰⁹⁻¹¹¹,
- cytotoxic ^{112,113},
- anti-ulcer ¹¹⁴⁻¹¹⁶,
- anti-emetic ¹¹⁷⁻¹¹⁹,
- anti-diabetic ¹²⁰⁻¹²³,
- hypoglycaemic ^{124,125},
- hypolipidaemic ^{88,124},

3 Classification and practice of Si Ni Tang

- anti-obesity¹²⁶⁻¹²⁸,
- anti-platelet^{129,130},
- antidependence property against chronic usage of morphine¹³¹,
- analgesic¹³²⁻¹³⁴ and
- cardiogenic properties¹³⁵⁻¹³⁷

Ginger is a strong antioxidant and may either mitigate or prevent the generation of free radicals^{85,138,139}.

3.1.5 *Cinnamomum cassia* Presl

Family: Lauraceae

Chinese name: Rou Gui

English name: Cinnamon

Used part: bark

Cinnamon is the oldest spice and has been used for centuries in many cultures for culinary purposes¹⁴⁰. In figure 6 the plant parts of *C. cassia* are shown. In TCM, *C. cassia* is commonly used for the treatment of dyspepsia, gastritis, blood circulation disturbances, inflammatory diseases¹⁴¹, fever, inflammation, chronic bronchitis and the induction of perspiration as well as for the improvement of blood circulation^{142,143}. According to TCM theory, Rou Gui is attributed to the kidney, spleen, heart and liver meridians. *C. cassia* supplements fire and strengthens yang, therefore it expels cold and alleviates pain, warms the channels to improve the blood circulation¹⁴⁴.



Figure 6. Plant parts of *C. cassia* (a) chromolithography plate of *C. cassia* from Köhler's Medicinal Plants (image: <http://de.wikipedia.org/wiki/Zimtkassie>), (b) stem and leaves (image: <http://online-media.uni-marburg.de/biologie/nutzpflanzen/antje/Info.html>), (c) Rou Gui, dried bark of *C. cassia* (image: K. Peter)

Several compounds have been isolated from *C. cassia* such as cinnamaldehyde, cinnamic acid, eugenol (see figure 7), coumarins, diterpenoids, and polyphenols^{145,146} which are the main characteristic components.

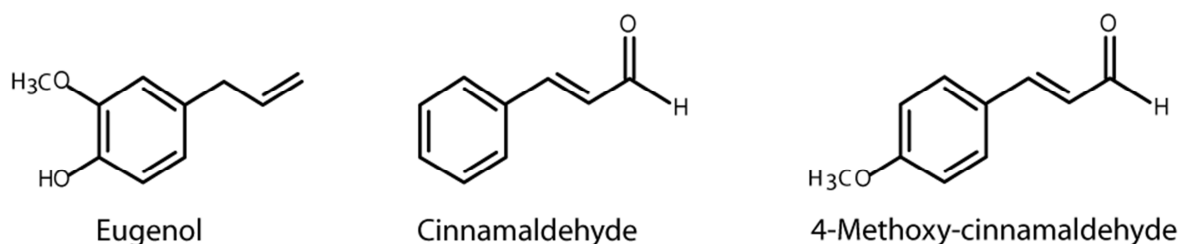


Figure 7. Chemical structure of eugenol, cinnamaldehyde and 4-methoxy-cinnamaldehyde

In addition, to its culinary use, cinnamon is gaining popularity because of its claim to health benefits, the most popular being its anti-diabetic properties^{140,147-149}.

Furthermore, several studies indicate a variety of bioactivities, including

- anti-oxidative¹⁵⁰⁻¹⁵²,
- anti-microbial¹⁵³⁻¹⁵⁵,
- hypotensive and peripheral vasodilatation¹⁵⁶,
- chemo-preventive¹⁵⁷⁻¹⁵⁹,
- anti-tumor¹⁶⁰⁻¹⁶³,
- anti-ulcer^{164,165},
- anti-gastric¹⁶⁶,
- anti-pyretic¹⁶⁷,
- platelet anti-aggregation^{145,168,169},
- anti-inflammatory¹⁷⁰⁻¹⁷² and
- protection of myocardia I/R-injury¹⁷⁰.

3.1.6 *Glycyrrhiza uralensis* Fisch

Family: Leguminosae

Chinese name: (Zhi) Gan Cao

English name: (pretreated) licorice

Used part: root

Licorice, one of the oldest and most popular herbal medicines in the world ¹⁷³, appears as a component herb in about 60 % of all TCM prescriptions ¹⁷⁴. In the food industry it is also very commonly used due to its sweet taste. In figure 8 the plant parts of *G. uralensis* are shown. In the theory of traditional Chinese medicine, Gan Cao is attributed to the heart, lung, spleen and stomach meridians. Furthermore Gan Cao is known as an emergency- and toxicity-moderating herb ¹⁷⁵ and for the ability to enrich qi, clear away heat and toxin, relieve spasm and to alleviate pain ¹⁴⁴.



Figure 8. Plant parts of *G. uralensis* (a) chromolithography plate of licorice from Köhler's Medicinal Plants (image: <http://de.wikipedia.org/wiki/Süßhölzer>), (b) leaves of *G. uralensis* (image: http://en.wikipedia.org/wiki/Glycyrrhiza_uralensis), (c) Gan Cao, dried Rhizoma Glycyrrhizae (image: K. Peter) (d) Zhi Gan Cao, processed and dried Rhizoma Glycyrrhizae (image: K. Peter)

After a long erroneous belief that glycyrrhizic acid is the only bioactive constituent, it was figured out that the flavonoids also contribute to the pharmacological activities of Gan Cao. Among the three hundred flavonoids isolated to date from *Glycyrrhiza* species ¹⁷⁶, flavanones, such as liquiritin and liquiritigenin, and chalcones such as isoliquiritin and isoliquiritigenin are

the major types. Together with the saponins they are supposed to be the components mainly responsible for the bioactivities of licorice¹⁷³ (see figure 9).

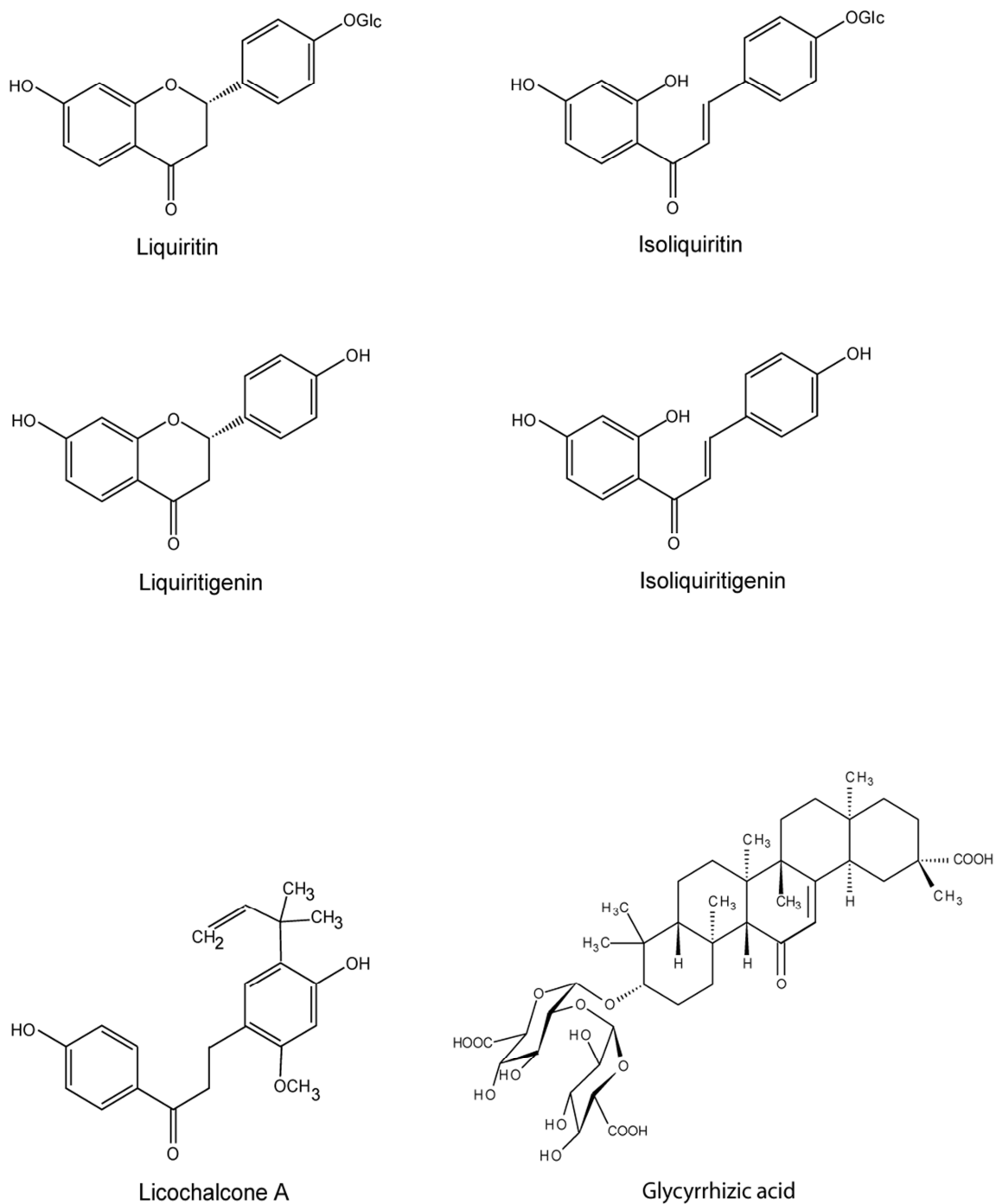


Figure 9. Chemical structure of liquiritin, isoliquiritin, liquiritigenin, isoliquiritigenin, licochalcone A and glycyrrhizic acid

The variety of Gan-Cao's biological activities include:

- anti-ulcer^{177,178},
- anti-oxidative¹⁷⁹⁻¹⁸¹,
- anti-inflammatory¹⁸¹⁻¹⁸⁶,
- a reduction of lipopolysaccharide-induced pro-inflammatory cytokine secretion^{187,188},
- anti-allergic¹⁸⁹⁻¹⁹¹,
- anti-viral¹⁹²⁻¹⁹⁵,
- anti-cancer¹⁹⁶⁻¹⁹⁸,
- anti-spasmodic¹⁹⁹⁻²⁰¹,
- anti-depressive^{202,203},
- anti-tumorigenic²⁰⁴⁻²⁰⁸,
- anti-microbial^{209,210},
- anti-diabetic²¹¹⁻²¹⁴,
- anti-thrombotic^{215,216},
- hepatoprotective²¹⁷⁻²²⁰,
- neuroprotective^{179,184,221,222},
- metabolic syndrome preventive activities^{213,223,224} and others.

It was discovered additionally that glycyrrhizic acid, a triterpene saponin and the main component of licorice, shows potential therapeutic properties for several virus diseases, such as chronic hepatitis B and C^{225,226} and human acquired immunodeficiency syndrome²²⁷. Experimental researches indicated that licorice had protection activity on the digestive system, respiratory system, nervous system, and endocrine system²²⁸.

3.2 The influence of traditional processing methods on the chemical profile of *A. carmichaelii* and interaction of plant constituents

3.2.1 Traditional processing of *A. carmichaelii*

A. carmichaelii is well-known worldwide for its wide application in Japan, Korea, India and China. However, its strong toxicity, due to diester diterpene alkaloids (DDAs) such as aconitine, hyaconitine and mesaconitine, has caused many fatal poisonings^{69,229,230}. The oral lethal dose for humans is a mere 1-5 mg of aconitine. The combination of two ester groups, an acetyl group at C₈ and a benzoyl ester group at C₁₄ (figure 10), are responsible for the high toxicity⁶⁹.

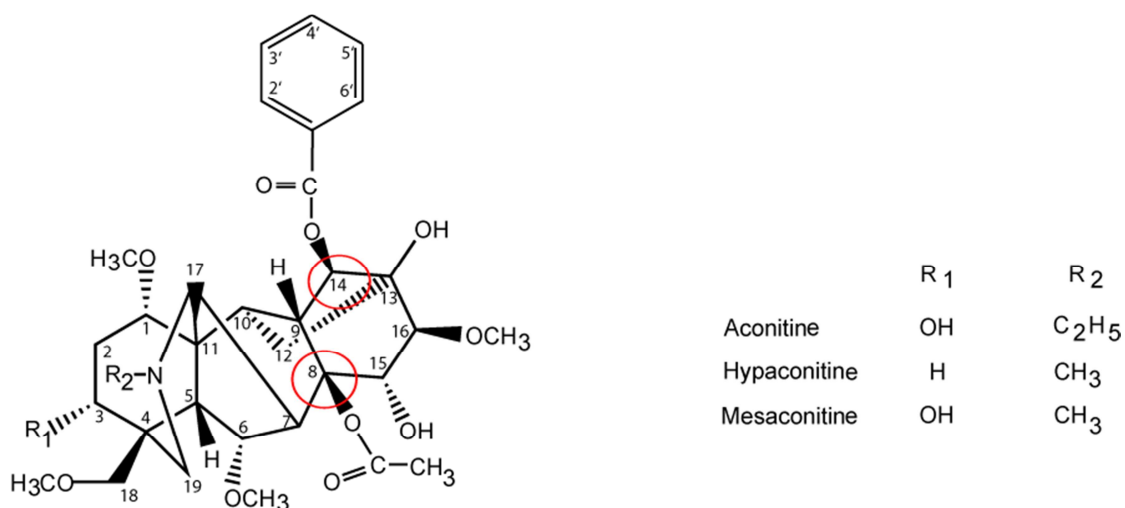


Figure 10. Chemical structure of aconitine, hyaconitine and mesaconitine, ester groups in red circle (C₈ and C₁₄)

The State Food and Drug Administration of China, states that only processed tubers and roots of *Aconitum* species are permitted to be applied orally and used in clinical decoctions. About seventy traditional and modern processing methods, such as steaming with salt-water or heating with sand²³¹, are used in the Chinese medicine to detoxify the tubers and roots of the *Aconitum* species⁷⁵. During the procedure, the DDAs are hydrolyzed and pyrolyzed into less toxic mono-diterpenoid alkaloids by replacing the acetyl group at C₈ of DDAs with a hydroxyl group²³². The toxicity of these so called benzoylaconines is 1/100-400 of aconitines. Further hydrolysis leads to the elimination of the benzoyl ester group at C₁₄, thus converted into aconines, a non-ester

diterpenoid with lower toxicity again ²³³ (figure 11). After the processing the alkaloid content is reduced by up to 90 % ^{234,235}. The LD₅₀ for unprocessed Fu Zi in mice, after oral administration is 5.49 g/kg and for processed Fu Zi 161 g/kg. The LD₅₀ for a decoction of unprocessed Fu Zi in mice is reported to be 26.3 g/kg ³⁷. In the Chinese medical journals 386 studies of aconitine poisoning comprising 2017 subjects were reported between 1989 and 2008 ²³⁶.

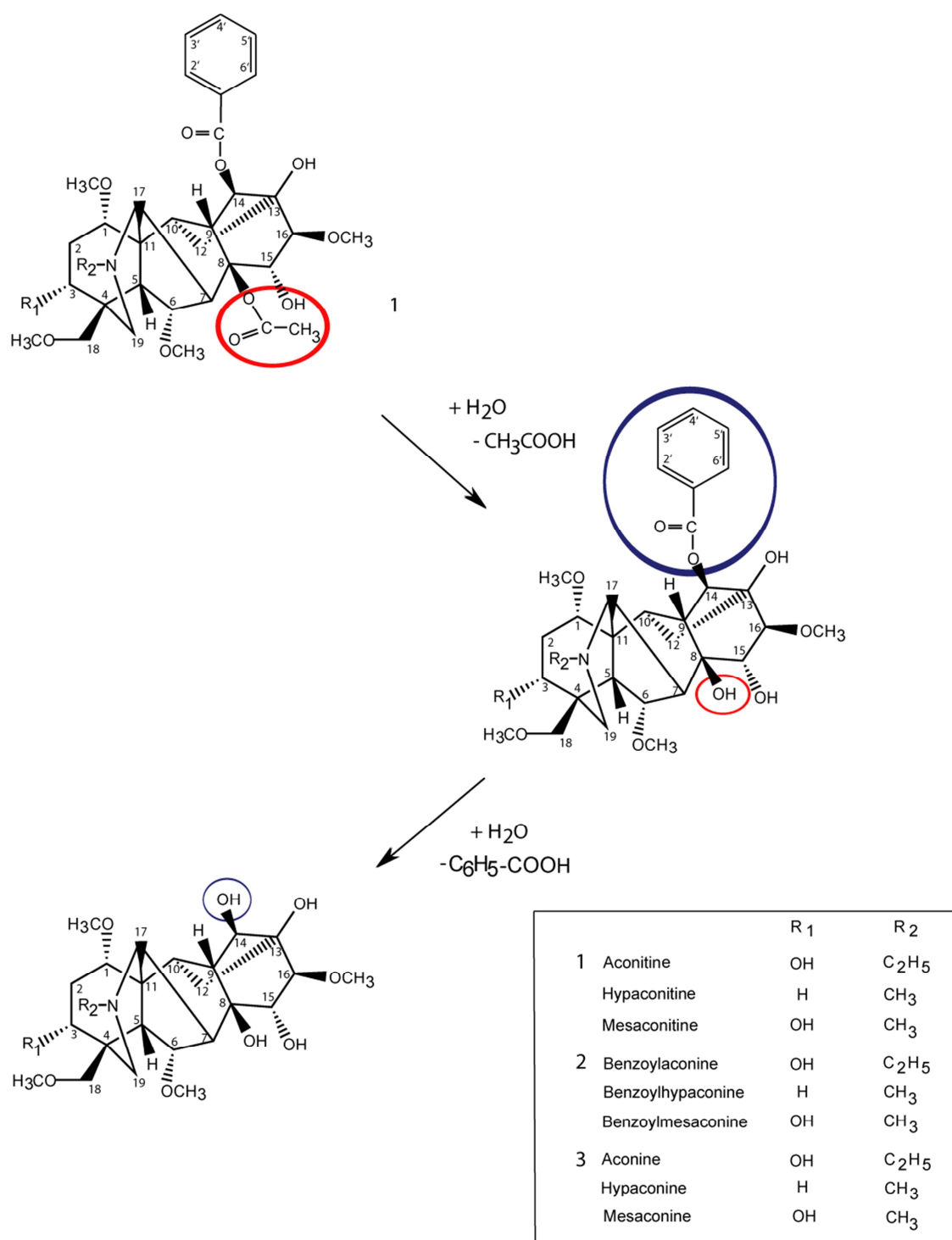


Figure 11. The detoxification of diterpene alkaloids by two-step hydrolysis

Lu et al. studied nine types of Fu Zi decoction samples, one raw and eight different processed forms, and quantified the amount of DDAs to assess the toxicity of each decoction sample. It was reported that *Heishunpian*, which is the most popular method of processing Fu Zi in China, showed the lowest quantity of aconitine, mesaconitine and hypaconitine compared with the other eight types of processed Fu Zi decoction samples⁶⁹.

Heishunpian means ‘black slices’. Sheng Fu Zi (raw aconite roots and tubers) are boiled. Ni Fu Zi, the boiled form, is then soaked in salt solution. The pieces are thoroughly boiled, rinsed with water, cut into slices, soaked in salt solution and again rinsed with water, the process being repeated until the slices turn black. They are then baked to half-dryness and finally completely dried in the sun⁷⁵.

3.2.2 Herb-herb interaction - detoxification

According to TCM theory, there are herbal combinations, which are declared to be safely paired in formulas, for example the *Aconitum* species together with *Radix Glycyrrhizae*.

Chen J. and Chen T. report in their book ‘Chinese Medical Herbology and Pharmacology’ that Fu Zi is basically used in combination with other herbs to increase the therapeutic effect and to minimize the potential side effects. Fu Zi is not recommended for use as a single herb in the treatment of cardiac disease, as it has only moderate potency, shows short duration and many serious side effects. Combined with *Radix Glycyrrhizae* (Gan Cao) and *Rhizoma Zingiberis* (Gan Jiang), the cardiotonic effect is increased, the duration of action is prolonged and, furthermore, with the addition of Gan Cao and Gan Jiang the side effects of Fu Zi can be reduced over fourfold³⁷.

Huang recently published his investigation into the ability of *Rhizoma Zingiberis* and *Radix Glycyrrhizae* to reduce the content of aconitine²³⁷. Investigations of Pei et al. produced similar results on the potency of licorice to diminish the toxicity of aconitine^{238,239}.

Gan Cao is well-known and generally used as a detoxifying herb in TCM. It neutralizes the toxicity of *Radix Aconiti Lateralis Praeparata*³⁷. The mechanism of this detoxifying process is reported to be an interaction between aconitine from aconite with glycyrrhizin (see figure 9,

section 3.1.6), the main compound of licorice. The two compounds form a complex which can be precipitated in the water solution²⁴⁰⁻²⁴².

However, Shen et al. found in his research, published in 2009, that liquiritin (see figure 9, section 3.1.6), a flavonoid of licorice, might be one of the components responsible for the alleviation of the toxicity of aconite²⁴³. Zhang and Yang reported similar conclusions on the ability of liquiritin to reduce the toxicity of *A. carmichaelii*²⁴⁴.

These results indicate the necessity to pre-treat aconite by standardized processing methods such as *Heishunpian* and also that the combination of the *Aconitum* species with *Radix Glycyrrhizae* and *Rhizoma Zingiberis* is essential for its effective therapeutic use and the reduction of side effects.

Licorice plays a special role in the detoxification process of aconite. It is important that further investigations are made to determine the mechanism of interaction between the compounds of licorice, ginger and cinnamon with the aconitines of aconite.

4 Material and methods

4.1 Quality control and analysis of the effective components

4.1.1 Plant material

Prof. Dr. Xu Riurong of the Shandong University of Traditional Chinese Medicine in China kindly provided several batches of processed and unprocessed roots of *A. carmichaelii* and *G. uralensis*, rhizome of *Z. officinale* and bark of *C. cassia* which had been collected in Shandong Province. All the herbs for *in vivo* experiments were collected in Hebei province and supplied by the Beijing International Institute of Biological Product, China.

Gan Cao (*G. uralensis*) was pretreated traditionally by roasting with honey. Fu Zi (*A. carmichaelii* root) was treated traditionally using the *Heishunpian* method (see section 3.2.1) in order to decompose the toxic diester diterpene alkaloids (DDAs).

4.1.2 Heavy metal experiments

4.1.2.1 Reagents

All reagents used, concentrated HNO₃ (65 % suprapur) and standard solutions of arsenic, lead, cadmium and mercury at a concentration of 1 mg/mL, were purchased at an analytical-reagent grade from Merck GmbH, Austria. Deionized water was produced using a water purification system (Elga Purelab Option 30, Veolia Water Systems, Austria).

4.1.2.2 Preparation of extracts

Five batches of each herb (*A. carmichaelii*, *Z. officinale*, *C. cassia* and *G. uralensis*) were tested. The raw herbal material was ground (50 g) and about 750 mg were accurately weighed into a Teflon® vessel. Next, 2 mL of deionized water and 7 mL of concentrated nitric acid were added to each vessel. Per run three samples of one batch and one blank sample were passed through a six-step program of the microwave reaction system, with maximum power 800 (see table 2). After cooling, the samples were quantitatively transferred into volumetric flasks and filled up to 25 mL with deionized water.

Preparation of standard solution for calibration

The elements were quantified against standard curves (0.5, 2, 5, 10, 20, 50 ppb), which were prepared on the day of analysis.

4.1.2.3 Instrumentation and conditions

A microwave reaction system, Multiwave 3000, Anton Paar, Austria was used to digest the ground herbs (La Moulinette 1000, Tefal, Germany).

Table 2. The following parameters were used for the microwave reaction system

Step	Power	Ramp	Hold	Fan
1	800 W	10:00 min	30:00 min	1
2	0 W	---	25:00 min	3

The Inductively Coupled Plasma–Optical Emission Spectroscopy (ICP-OES) was used for the determination of possible heavy metal contamination. These investigations were performed on a Thermo iCAP 6300 duo ICP Spectrometer, Thermo Scientific, Germany with an iTEVA Analyzer, Thermo Scientific Electron Corporation, Germany.

The optimized ICP-OES parameters were used:

- Sample flush time: 30 s
- Flush pump rate: 25 rpm
- Analysis pump rate: 50 rpm
- Pump stabilization time: 5 s
- RF power: 1300 W
- Nebulizer gas flow rate: 0.16 MPa
- Auxiliary gas flow rate: 0.5 L/min
- Number of repeats: 3

Validation of the method

The accuracy of the analytical procedure was proofed by triplication of the samples. Furthermore, the results were double-checked and confirmed by PMA, Peri Medizinische

Analytik, Sindelfingen, Germany. Analytical validation was implemented by analysing spiked samples. Therefore different amounts of reference standards As, Pb, Cd and Hg were added to the ground raw herbal material prior to microwave digestion. The calculation was done according to the equation: recovery [%] = [amount of element - amount of element in plant material] / amount of element spiked * 100 according to AOAC Guidelines²⁴⁵. Recoveries from analyses of reference materials in spiked samples were in the range of 91-107 %.

The blanks were prepared without a sample, followed by analysing them under the same experimental steps and conditions. Blank experiments were carried out for each batch and each procedure. Limit of quantification (LOQ) using ICP-OES were 0.02 mg/kg for As, 0.2 mg/kg for Pb, 0.01 mg/kg for Cd and 0.05 mg/kg for Hg.

4.1.3 HPLC experiments

4.1.3.1 Reagents

Analytical grade methanol, acetonitrile, ethanol, and hydrochloric acid (Merck GmbH, Austria) were used for the extraction and preparation of standard solutions. HPLC grade acetonitrile, *ortho*-phosphoric acid, tetrabutylammonium hydroxide and water were purchased from Fisher Scientific, Austria.

Chemical standards of aconitine, mesaconitine, hypaconitine, liquiritin, isoliquiritin, liquiritigenin, isoliquiritigenin, licochalcone A, 8-gingerol and 6-shogaol were purchased from PhytoLab GmbH, Germany. Eugenol, cinnamaldehyde, 4-methoxy-cinnamaldehyde and glycyrrhizic acid were purchased from Lactan GmbH & Co. KG, Austria. The identity and purity of the following chemicals were further validated by HPLC-DAD: aconitine (95.2 %), hypaconitine (96.2 %), mesaconitine (98.8 %), glycyrrhizic acid (97.9 %), liquiritin (96.7 %), isoliquiritin (97.8 %), liquiritigenin (99.8 %), isoliquiritigenin (99.9 %), licochalcone A (99.0 %), 8-gingerol (96.2 %), 6-shogaol (98.6 %), eugenol (99.5 %), cinnamaldehyde (95.8 %) and 4-methoxy-cinnamaldehyde (96.4 %). These reference standards were used without further purification.

4.1.3.2 Preparation of extracts

Water extraction/decoction. The sliced and dried roots, pretreated *G. uralensis*, pretreated *A. carmichaelii* roots, *Z. officinale* roots and *C. cassia* (8:6:3:1) were soaked in 400 mL of cold water for one hour in a clay pot. Then the mixture was brought to boiling point and heated gently for 30 min. The herbal mixture was re-extracted to soak for another 30 min in 400 mL of cold water and the two filtered extracts were then combined and centrifuged at 8.000 rpm for 40 min at 4 °C (Sorvall RC-5C Plus, GMI, Minnesota, USA). The same procedure, amount and concentration, as described above, were applied respectively for the decoction of single herbs or different combinations of herbs. Prior to lyophilization (Christ-Loc-1m Alpha 1-4 Rieger, Christ, Austria) all samples were filtered through a 0.45- μ m sterile syringe filter (Filtropur, Sarstedt, Germany) and freeze-dried in liquid nitrogen. Each remaining powder was weighed precisely and dissolved in 1 mL of water before being subjected to the HPLC-system. The lyophilization was an important process, enabling us to produce high-concentrated sample solutions.

Extraction with organic solvents. The unprocessed and processed *A. carmichaelii* roots were pulverized (La Moulinette, Moulinex) and passed through a 24-mesh sieve. Extraction of powdered samples was performed using the method of G. Lu et al.⁶⁹. The amount of 0.5 mg of the ground root of processed and unprocessed *A. carmichaelii* was extracted in 5 mL of four types of solvents (methanol, methanol-water 1:1, water, 1 % hydrochloric acid) in order to determine the most effective extraction medium. Each mixture was extracted for 60 min by sonification (Sonorex Super Rk 106 ultrasonic processor, Bandelin, Germany) at room temperature and then centrifuged at 3000 rpm for 10 min. The supernatant of each sample was concentrated under vacuum and the residues were finally dissolved in 1 mL of the corresponding extraction solvent. The average value of the DDAs in samples with extracting agents were calculated and specified as their amounts in a particular solid sample and the results compared to find the best extraction agent. All extractions were carried out in triplicates. Each sample was filtered through a 0.2- μ m sterile syringe filter (Filtropur, Sarstedt, Germany) prior to the examination by HPLC.

4.1.3.3 Instrumentation and conditions

An Agilent 1100 series LC system consisting of a binary pump, a vacuum degasser, an autosampler and an UV-VIS (ultraviolet-visible) diode array detector (DAD) were applied. Two simple and quick methods were developed and were carried out on a Hypersil BDS RP C-18 column (100 x 3 mm; particle size 3 μ m; Thermo Scientific, Germany) at 25 °C. The injection volume was 5 μ L. Separation of alkaloids was optimized using a gradient system of an aqueous buffer (A) containing 15 mM *ortho*-phosphoric acid and 1.5 mM tetrabutylammoniumhydroxide and acetonitrile (pH 3) (B), developed by Reisch et al.²⁴⁶ and at a flow rate of 0.5 mL/min.

For the gradient elution of the mobile phase see table 3. The DAD detector signal was set at 233 nm with the reference wavelength of 550 nm. The UV spectra were acquired from 200 to 400 nm.

Table 3. The gradient elution of the mobile phase used for the separation of the alkaloids

Time	Gradient	Eluent
0-11 min	5-24.4 %	B
11-17 min	24.4-26 %	B
17-18 min	26-40 %	B
18-18.1 min	40-100 %	B
18.1-23 min	100 %	B

For the qualitative fingerprinting analysis of SNT decoction successful separation was achieved using the same eluents mentioned above and a gradient elution of the mobile phase shown in table 4. The DAD detector signal was set at 240 nm with the reference wavelength of 550 nm.

Table 4. The gradient elution of the mobile phase used for the analysis of SNT decoction

Time	Gradient	Eluent
0-10 min	10-20 %	B
10-25 min	20-25 %	B
25-50 min	25-50 %	B
50-50.1 min	50-100 %	B
50.1-55 min	100 %	B

Preparation of standard solutions for the calibration

Aconitine 1.60 mg, hypaconitine 2.30 mg and mesaconitine 1.50 mg were accurately weighted and dissolved with exact 1 mL of acetonitrile/H₂O (80:20). The combination and dilution of defined volumes of these solutions gave a mixed stock solution containing 160 µg aconitine, 230 µg hypaconitine and 150 µg mesaconitine per mL acetonitrile/H₂O (80:20). Calibration curves were established for each alkaloid, based on ten concentrations of each alkaloid (with a range of 2.00-160.00 µg/mL for aconitine, 2.88-230.00 µg/mL for hypaconitine and 1.88-150.00 µg/mL for mesaconitine) by diluting the stock solution with acetonitrile/H₂O (80:20) in appropriate quantities (table 5).

Table 5. Concentration [µg/mL] of the calibration standards for aconitine, hypaconitine and mesaconitine.

Standard	Dilution (µg/mL)									
	A	B	C	D	E	F	G	H	I	J
Aconitine	2.00	4.00	6.00	8.00	12.00	16.00	32.00	40.00	80.00	160.00
Hypaconitine	2.88	5.75	8.53	11.50	17.25	23.00	46.00	57.50	115.00	230.00
Mesaconitine	1.88	3.75	5.63	7.50	11.25	15.00	30.00	37.50	75.00	150.00

The limit of detection (LOD) was defined as the sum of the mean value of the highest peak in ten tested blanks and the threefold value of the standard deviation of the blank test, whereas the limit of quantification (LOQ) was defined as the tenfold value of the standard deviation of the blank test.

Validation of extraction and HPLC method

Repeatability. The repeatability was determined in parallel tests by extracting five independent samples of unprocessed ground roots of *A. carmichaelii* with methanol-water (1:1) prior to the analysis by HPLC-DAD.

Precision. To determine the intra-daily precision of the analytical method, the same extract used for the analysis of repeatability was subjected five times to HPLC-DAD analysis on the same day. For inter-daily precision, measurements twice a day on three consecutive days were conducted. The content of aconitine, hypaconitine and mesaconitine of the samples were

analyzed, and the relative standard deviation (RSD) within the measurements was used for the evaluation of precision and repeatability according to Csupor D. et al.²⁴⁷.

Recovery. The recovery was accomplished by adding different amounts of aconitine (24 µg/mL), hypaconitine (92 µg/mL) as well as mesaconitine (22.5 µg/mL) to the ground unprocessed herbal material and subsequent extraction. The calculation was made according to the equation: recovery [%] = [amount of alkaloid - amount of alkaloid in plant material] / amount of alkaloid spiked * 100 according to AOAC Guidelines²⁴⁵.

Preparation of standard solution of aconitine, hypaconitine and mesaconitine

A standard solution of VDR1, containing 52 µg aconitine, 50.7 µg hypaconitine and 44.7 µg mesaconitine per mL, was used for the analysis of herbal interaction and for the identification of the DDAs in all other samples.

Herbal interaction within decoction

Each herb (*G. uralensis*, *C. cassia*, *Z. officinale*) was combined with the roots of unprocessed *A. carmichaelii* and the decoction prepared according to the description in section 4.1.3.2. The concentration of the herbal mixture was corresponding to the original compounding in the SNT-formula (see table 6).

Table 6. Combinations of herbs for decoction to analyze possible herbal interaction

Herb	+	Herb	+	Herb	Ratio ^a
unprocessed <i>A. carmichaelii</i>		--		--	6
unprocessed <i>A. carmichaelii</i>		<i>G. uralensis</i>		--	6:8
unprocessed <i>A. carmichaelii</i>		<i>C. cassia</i>		--	6:1
unprocessed <i>A. carmichaelii</i>		<i>Z. officinale</i>		--	6:3
unprocessed <i>A. carmichaelii</i>		<i>C. cassia</i>		<i>Z. officinale</i>	6:1:3

^aSame ratio as in SNT

Interaction between single compounds and the DDAs

To determine the interaction among single compounds and the DDAs, 100 µL of a stock solution (1 mg/mL) of the bioactive components, known to be present in the used parts of three plants contained in SNT (*G. uralensis*: glycyrrhizic acid, liquiritin, isoliquiritin, liquiritigenin,

isoliquiritigenin, licochalcone A; *Z. officinale*: 8-gingerol, 6-shogaol, and *C. cassia*: eugenol, cinnamaldehyde and 4-methoxy-cinnamaldehyde) were combined individually with 15 μl of VDR1 (stock solution of DDAs).

To analyze the concentration of isoliquiritin needed to have an effect on the reduction of aconitine, hyaconitine and mesaconitine, the quantity of 15 μl of VDR1 were mixed with different amounts of the stock solution of isoliquiritin (20, 30, 40, 50, 100 μl) and filled up with deionized water to 115 μl prior to the injection into the HPLC system.

4.1.4 NMR experiments

4.1.4.1 Reagents

The deuterated solvents used for nuclear magnetic resonance (NMR) spectroscopy, D_2O with a deuteration grade of $\text{D} \geq 99.90\%$ and CD_3OD , deuteration grade of $\text{D} \geq 99.80\%$, were purchased from Eurisotop, Germany and were used without further purification. The reference standards, liquiritin, liquiritigenin, isoliquiritin, isoliquiritigenin and aconitine were purchased from PhytoLab GmbH, Germany.

4.1.4.2 Preparation of extracts

For the experiments on the interaction between aconitine and liquiritin, the stoichiometry was determined using the method of continuous variations (Job's method)²⁴⁸⁻²⁵⁰. Nine samples of mixtures of host and guest were prepared, and the total concentration was kept constantly at 500 μM . With a focus on keeping the analysis as practice-orientated as possible we started the solubility test with deuterated water (D_2O) and added deuterated methanol (MeOD) gradually. The best solubility was achieved using $\text{D}_2\text{O}/\text{MeOD}$ in the concentration of 2:1.

Stock solutions of aconitine (500 μM) and liquiritin (500 μM) in $\text{D}_2\text{O}/\text{MeOD}$ (2:1) were prepared separately. For each aconitine/liquiritin system, nine 5 mm high precision NMR sample tubes (Promochem, Wesel, Germany) were filled with 0.7 mL of aconitine and liquiritin in the following ratios (see table 7): $[\text{aconitine}]/([\text{aconitine}] + [\text{liquiritin}]) = 0.0, 0.13, 0.26, 0.40, 0.50, 0.60, 0.73, 0.87, \text{ and } 1.0$, where [aconitine] and [liquiritin] represent the initial respective concentrations of the aconitine and liquiritin.

Table 7. Concentration of aconitine and liquiritin for Job's plot experiment

Concentration Aconitine (μM)	Concentration liquiritin (μM)	Mole fraction aconitine	Mole fraction liquiritin
0	500	0	1
67	433	0.13	0.87
133	367	0.27	0.73
200	300	0.4	0.6
250	250	0.5	0.5
300	200	0.6	0.4
367	133	0.73	0.27
433	67	0.87	0.13
500	0	1	0

The $\text{C}_6\text{-H}$ signal of liquiritin (6.44 ppm) (see figure 12) was observed to calculate $\Delta\delta \cdot [\text{liquiritin}]$, where $\Delta\delta$ is the shift (ppm) of the $\text{C}_6\text{-H}$ signal of liquiritin induced by the addition of aconitine. Values for $\Delta\delta \cdot [\text{liquiritin}]$ were plotted against the aconitine-mole fraction, $[\text{aconitine}]/([\text{aconitine}] + [\text{liquiritin}])$.

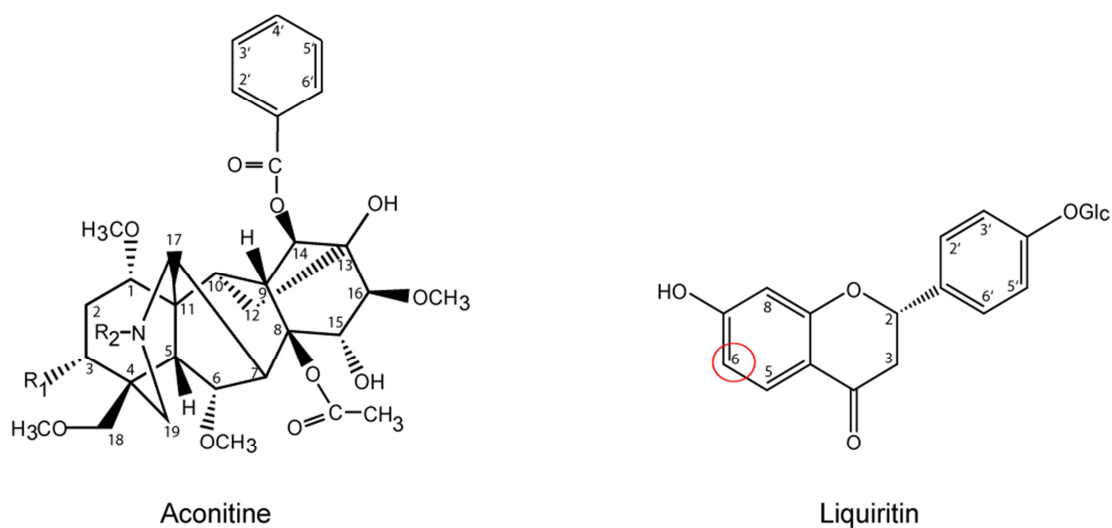


Figure 12. Chemical structure of aconitine and liquiritin, red circle: $\text{C}_6\text{-H}$ of liquiritin was observed in Job's Plot analysis

^1H -NMR titrations²⁵¹ were performed to evaluate the association constant K . Stock solutions of liquiritin (1.4 mM) and aconitine (4.6 mM) in $\text{D}_2\text{O}/\text{MeOD}$ (2:1) were prepared separately. Ten NMR tubes were filled with different concentrations of aconitine: 0.0, 0.1, 0.2, 0.4, 0.8, 1.2, 1.6, 2.0, 3.0, 4.0 mM and a constant concentration of liquiritin (0.2 mM) in a 0.7 mL total solution of each sample. $\Delta\delta_{\text{obs}}$ was plotted against the concentration of aconitine, where $\Delta\delta_{\text{obs}}$ is the difference between the ^1H -NMR chemical shift of liquiritin and the ^1H -NMR chemical shift of free liquiritin that was observed. The same analysis was repeated inversely with a constant concentration of aconitine and a varying concentration of liquiritin. In this way, stock solutions of aconitine (1.4 mM) and liquiritin (4.6 mM) in $\text{D}_2\text{O}/\text{MeOD}$ (1:2) were prepared separately and filled in ten NMR tubes as described above. The liquiritin concentration remained constant (0.2 mM) in a 0.7 mL total solution of each sample, whereas the concentration of aconitine varied: 0.0, 0.1, 0.2, 0.4, 0.8, 1.2, 1.6, 2.0, 3.0, 4.0 mM. A curve-fitting²⁵²⁻²⁵⁴ was carried out to show fast exchange systems^{255,256}:

$$\Delta\delta_{\text{obs}} = 0.5 \Delta\delta_{\text{max}} \frac{[(H + G + (1/K)) - [(H + G + (1/K))^2 - 4HG]^{1/2}]}{H}$$

H : constant concentration of compound in each sample in mol/L

G : variable concentration of compound in each sample in mol/L

K : association constant in L/mol

For 2D ROESY (Rotating-frame Overhauser Spectroscopy), one sample solution of aconitine (500 μM) and liquiritin (500 μM) in 0.7 mL $\text{D}_2\text{O}/\text{MeOD}$ (2:1) was prepared.

4.1.4.3 Instrumentation and conditions

All spectra were recorded on a Bruker DRX-600 AVANCE spectrometer (Bruker, Rheinstetten, Germany) at 600.13 MHz (^1H) and the data compiled using the Bruker Topspin 2.1 software. All samples were measured at 298.1 $\pm$ 0.1 K.

All spectra were corresponded to the CH_3 group signal of internal acetate (δ ^1H : 2.080 ppm) which was present in ca. 200 μM concentrations. Its shift was not influenced by the presence of aconitine or liquiritin as the shift difference of 87 Hz to external acetone (δ ^1H : 2.225 ppm) remained constant.

To determine the 2D homonuclear ROESY spectrum, 512 experiments with 2048 data points and 32 scans were carried out. Each one was recorded using a standard Bruker program. The water signal was suppressed by presaturation. A mixing time of 400 ms and a relaxation delay of 1.5 s were used. After linear forward prediction to 1024 data points in the f_2 dimension and sinusoidal multiplication in both dimensions, Fourier was transformed in both dimensions resulting in a 2D-spectrum with a range of 6000 Hz in both dimensions.

4.1.5 UV-VIS experiments

4.1.5.1 Reagents

HPLC grade acetonitrile was purchased from Fisher Scientific, Austria. D₂O with a deuteration grade of $D \geq 99.90\%$ and CD₃OD with a deuteration grade of $D \geq 99.80\%$, were purchased from Eurisotop, Germany. The reference standards, liquiritin and aconitine, were purchased from Phytolab, Germany.

4.1.5.2 Preparation of extracts

The samples described in section 4.1.4.2 were used again and further diluted with the tenfold quantity of D₂O/MeOD (2:1).

4.1.5.3 Instrumentation and conditions

For the instrumentation used see section 4.1.3.3. The amount of 5 μ L of each sample was directly injected into the DAD of the HPLC system (without any separation by column) and washed with 100 % acetonitrile at a flow rate of 0.25 mL/min. The DAD detector signal was set at 233 nm with the reference wavelength of 550 nm.

4.2 In vivo experiments

4.2.1 Drugs and reagents

According to an ancient traditional formula, Si Ni Tang (SNT) is composed of parts of three herbs, the radix of *Aconitum carmichaelii* Debx. (aconite, Zhi Fu Zi), the rhizoma of *Zingiber officinale* Ross. (ginger, Gan Jiang) and the radix of *Glycyrrhiza uralensis* Fisch. (licorice, Zhi Gan Cao). We modified the mixture by adding the bark of *C. cassia* (cinnamon, Rou Gui). All herbs for *in vivo* experiments were supplied by the Beijing International Institute of Biological Product, and had been tested in accordance with the standard of quality control of Chinese National Pharmacopoeia (2010).

Preparation of decoction. The dried herbs were soaked in cold water for one hour, then heated to boiling point and cooked gently for 30 min. The herbal mixture was re-extracted by heating for a further 30 min. Both extracts were combined, filtered and diluted to the calculated concentration appropriate to the surface area of the rats. The dose for the low dosage group (4.5 g/kg) was conformable to the generally used daily dose for a Chinese patient and for the high dosage group (13.5 g/kg) the dose was calculated to be three times as much. Extract of SNT was kindly provided by Xi Yuan Hospital in Beijing. Monopril, Fosinopril sodium 10 mg/tablet, was supplied by Sino-American Shanghai Squibb Pharmaceutical Co., Ltd., batch number 200808091. ELISA kits were purchased by R&D systems, USA (tumor necrosis factor- α (TNF α) batch number: E10H2602; interleukin-6 (IL-6) batch number: E10H2601; high sensitivity C-reactive protein (hs-CRP) batch number: E10H2603; transforming growth factor- β 1 (TGF- β 1) batch number: DZE30636; collagen I (col-I) batch number: DZE30413; collagen III (col-III) batch number: DZE30458) and GBD Ltd., USA (atrial natriuretic peptide (ANP) batch number: A056-10; total nitric oxide (NO) batch number: N022-10; cardiac troponin T (cTNT) batch number: C123-10; endothelin 1 (ET-1) batch number: E062-40).

4.2.2 Instrumentation

Electrocardiograph: HDI Philips Netherlands ultrasound diagnosis, CL15-7 Hf array detector (7-15 MHz).

Photometer: Multiskan MK3, Thermo Labsystems, Thermo Scientific, Netherlands.

Shaker: Thermo-Shaker, MB100-4P, Hangzhou Allsheng Instruments Co., Ltd. China.

Homogenizer: Ultra Turrax T18 basic, IKA-Works, Guangzhou, China.

Microscope: BH2, Olympus Japan.

DpxView Por Software micro-color image processing system, DeltaPix corporation.

4.2.3 Animal models, grouping and medication

The Sprague Dawley rats were purchased from the Beijing Vital River Laboratory Animals Technology Co., Ltd. and acclimatized for three days before being used for experiments. All animals received care in compliance with the guidelines outlined in the Guide for the Care and Use of Laboratory Animals published by the U.S. National Institutes of Health (No. 85-23, revised 1996). Acute myocardial infarction (AMI) was induced in both male and female rats (180–200 g) by left anterior descending artery (LAD) ligation. The surgical procedures were performed on the first day by two skilled operators using the LAD ligation technique by Pfeffer et al.²⁵⁷. Each rat was placed in a supine position on a table and its extremities were fixed to four electrocardiography leads on the table. The left side of sternum and abdomen were disinfected and sheared to prepare for the operation. A left parasternal incision was made through the third and fourth ribs and the pericardium was gently opened to expose the heart. The heart was positioned in a way that the left ventricle (LV), aorta and left atrium were exposed for suture placement. A 3-0 suture was placed in the 1/3 anterior myocardium to occlude the LAD. The heart was returned to its original position. The sternum and skin incision was closed with 7-0 sutures. The rats of the control group underwent a sham operation, consisting of thoracotomy and cardiac exposure but without coronary artery ligation. Infarct rats were randomly assigned to four groups (see table 8). The oral administration of the drugs began on the day following the AMI-induction. SNT (4.5 or 13.5 g/kg) and Fonosinopril (900 µl/kg), diluted with distilled water were administered orally in a volume of 10 mL/kg body weight once every morning for 30 days. The animals were weighed every five days and prior to death. Treatment with a low dose of SNT

and Fosinopril (FPS), an angiotensin-converting-enzyme inhibitor, was calculated with the aid of a conversion table corresponding to the daily dose generally used for humans and converted to the surface area of the rats. The SNT-High Dose (SNT-HD) concentration was calculated to be three times higher than that of SNT-Low Dose (SNT-LD). Drinking water was administered orally to the Sham-operated (SH-O) and model group. A total of six male and six female rats per group survived in the operation. Standard diet was withheld the day prior to the operation while water was provided freely.

Table 8. The six experimental groups for examining the effects of SNT on early ventricular remodeling in rats post- myocardial infarction in vivo. Drugs diluted with distilled water were administered orally once a day for 30 days starting one day after induction of AMI.

Group	N	Operation	Oral administration
Normal	12	-	-
SH-O	12	Sham-operation	Drinking water
Model	12	AMI	Drinking water
FPS	12	AMI	Fosinopril (900 µg/kg)
SNT-LD	12	AMI	Low dose SNT (4.5 g/kg)
SNT-HD	12	AMI	High dose SNT (13.5 g/kg)

4.2.4 Echocardiography

Transthoracic echocardiographic studies were performed 31 days after surgery. Ten rats from each group were anesthetized by intraperitoneal injection of ethyl carbamate (6 mL/kg) (Qingdao Fraken International Trading Co., Ltd., China), placed on a table in a supine position and their extremities fixed to four electrocardiography leads on the table. The chest was shaved and further cleaned with a chemical hair remover to maximize the ultrasound reception. Aquasonic gel (Parker Laboratories Inc., USA) was applied to the thorax surface to optimize the visibility of the cardiac chambers. The values were calculated on an average of five cycles. From the cardiac short axis (papillary level), left ventricle end-diastolic dimension (LVDd) and left ventricle end-systolic dimension (LVDs) were obtained. The ejection fraction (EF) was calculated from the left ventricle end-diastolic volume (LVEDV) and the left ventricle end-systolic volume (LVESV) as

the following: $EF = LVEDV - LVESV / LVEDV$. A scientist with no pre-knowledge of the experiment, performed the echocardiographic analysis and calculation.

4.2.5 Blood sampling and tissue collection

After echocardiographic measurements, the anesthetized rats were put to death. Blood samples were taken with a heparinized syringe from the LV cavity and centrifuged at 3000 rpm for 15 min. The plasma and serum were conserved at -80 °C for further analysis. The hearts were removed from the chest and weighed. Two transverse sections of the hearts were made from the apex to the base. One section of each heart was embedded in 10 % neutral buffered formaldehyde solution for pathohistological analysis and the other section was snap-frozen in liquid nitrogen for enzyme-linked immunosorbent assay (ELISA). The livers were removed for toxicological analysis and fixed in 10 % neutral formaldehyde at 4 °C. The organ samples were cut into 4 µm section and stained with hematoxylin and eosin.

4.2.6 Assay of serum and heart tissue levels by ELISA

Five of the twelve frozen heart tissue samples of each group were randomly picked for ELISA tests. The heart sections were weighed and 300 mg of each sample were cut into small pieces and 1 mL of saline solution was added. After homogenization on ice, samples were centrifuged and the supernatants were stored at -80 °C for analysis. ELISA tests were performed strictly according to the instructions. The protein concentration of each tissue sample was measured with the BCA (bicinchoninic acid) Protein Assay Kit by ELISA.

The following markers, known to be involved in the process of cardiac remodeling, were determined by ELISA:

Collagen markers (measured in serum and heart tissue): collagen I, collagen III and transforming growth factor-β1

Functional index (serum): atrial natriuretic peptide and cardiac troponin T

Inflammatory markers (serum): interleukin-6, interleukin-1β, tumor necrosis factor-α and high sensitivity C-reactive protein

Vascular active markers (serum): endothelin-1 and nitric oxide

4.2.7 Statistical analysis

All results were tested on normal distribution by aid of ‘One-Sample Kolmogorov-Smirnov Test’. Unless otherwise specified, the results were tabulated and presented as the mean $\pm$ standard error of the mean, and the significance of changes was assessed with the aid of one-way repeated measures analysis of variance (ANOVA) followed by Bonferroni’s Holm test for multiple comparisons. One-way ANOVA Tukey HSD test was used for pairwise multiple comparisons. A P-value of less than 0.05 was considered to be statistically significant. Data was analyzed using ‘The Statistical Package for the Social Sciences’, version 17 (SPSS Software, SPSS Inc., Chicago, USA).

5 Results

5.1 Quality control and analysis of the effective components

5.1.1 Heavy metal tests

Table 9 and figure 13 show the concentrations of the ions, arsenic (As), lead (Pb), cadmium (Cd) and mercury (Hg) analyzed in the twenty-four batches of the following medicinal herbs: the pretreated radix of *Aconitum carmichaelii*, the rhizoma of *Zingiber officinale*, the cortex of *Cinnamomum cassia* as well as the untreated and pretreated radix of *Glycyrrhiza uralensis*.

pretreated *A. carmichaelii*, *Z. officinale*, *C. cassia* as well as untreated and pretreated *G. uralensis*. Twenty batches (batches number 1 to 4 of each herb) were collected in Shandong Province and were kindly provided by Prof. Dr. Xu Riurong of the Shandong University of Traditional Chinese Medicine, China. The batches number 5, collected in Hebei province and kindly supplied by the Beijing International Institute of Biological Product, were also used for *in vivo* experiments.

The limits of quantification (LOQ) using ICP-OES were 0.02 mg/kg for As, 0.2 mg/kg for Pb, 0.01 mg/kg for Cd and 0.05 mg/kg for Hg. The relative standard deviation (RSD) for each sample was below 5 %. Most batches contained detectable levels of As, Pb and Cd, only batch number 3 of *C. cassia* showed an As level below the LOQ and batch number 5 of the pretreated *G. uralensis* contained Pb levels below the LOQ. Mercury was not detected in any of the samples. Arsenic and lead were measured at levels of 1.60 mg/kg and 2.00 mg/kg respectively in *A. carmichaelii*. Cadmium was measured at a level under 0.72 mg/kg in *C. cassia*. In the samples used for the decoction of *in vivo* experiments (batch number 5 of each herb) nearly all concentrations of detected elements were noticeably lower, partly considerably lower than in the batches from Shandong Province. Only the Cd level of *C. cassia* was higher, at 0.72 mg/kg whereas the other batches of *C. cassia* contained Cd levels from 0.27 – 0.49 mg/kg and the Cd level of *A. carmichaelii* which was, at 0.08 mg/kg, similar to the other four batches of *A. carmichaelii* (0.04 - 0.08 mg/kg).

The element concentrations of the four batches (batch number 1 – 4) from Shandong Province show various remarkable differences within each group of herb (see table 9). It is noticeable that

in general the levels of As and Pb in *A. carmichaelii* are conspicuously high (1.6 - 1.3 mg/kg) compared to the concentrations in other tested herbs, whereas in *C. cassia* the As amount is extremely low (< 0.02 – 0.05 mg/kg). In comparison with the Cd levels of *A. carmichaelii* (0.04 – 0.08 mg/kg) und *G. uralensis* (0.01 – 0.03 mg/kg), the Cd concentrations of *C. cassia* (0.28 – 0.47 mg/kg) and *Z. officinale* (0.27 – 0.55 mg/kg) were found to be considerably higher.

Table 9. Concentration of arsenic, lead, cadmium and mercury in the dried, grinded medicinal herbs

		Concentration mg/kg			
		Arsenic	Lead	Cadmium	Mercury
Upper limit ^a		--	5.00	1.00	0.10
Medicinal herb	Batch number ^b				
<i>A. carmichaelii</i> , pretreated	1	1.30	2.00	0.04	< 0.05
	2	1.40	2.00	0.06	< 0.05
	3	1.50	2.00	0.08	< 0.05
	4	1.60	1.80	0.06	< 0.05
	5 ^c	1.00	1.50	0.08	< 0.05
<i>Z. officinale</i>	1	0.48	0.80	0.55	< 0.05
	2	0.72	0.40	0.27	< 0.05
	3	0.60	0.40	0.28	< 0.05
	4	0.54	0.40	0.31	< 0.05
	5 ^c	0.41	0.40	0.14	< 0.05
<i>C. cassia</i>	1	0.04	0.80	0.30	< 0.05
	2	0.03	0.40	0.28	< 0.05
	3	< 0.02	0.40	0.47	< 0.05
	4	0.05	0.80	0.29	< 0.05
	5 ^c	0.04	0.40	0.72	< 0.05
<i>G. uralensis</i> , pretreated	1	0.53	0.60	0.02	< 0.05
	2	0.44	0.50	0.01	< 0.05
	3	0.48	0.50	0.02	< 0.05
	4	0.73	0.70	0.03	< 0.05
	5 ^c	0.15	< 0.2	0.01	< 0.05
<i>G. uralensis</i> , untreated	1	0.56	0.60	0.02	< 0.05
	2	0.54	0.50	0.02	< 0.05
	3	0.46	0.50	0.02	< 0.05
	4	0.79	0.90	0.02	< 0.05

^a the recommended upper limit according to the European Pharmacopoeia ²⁵⁸.

An upper limit for As has not yet been defined

^b batch number (indicates different batches)

^c these batches were used for *in vivo* experiments

n = 3; the relative standard deviations (RSD) for each sample was below 5 %; < 0.05 = below the LOD

5 Results

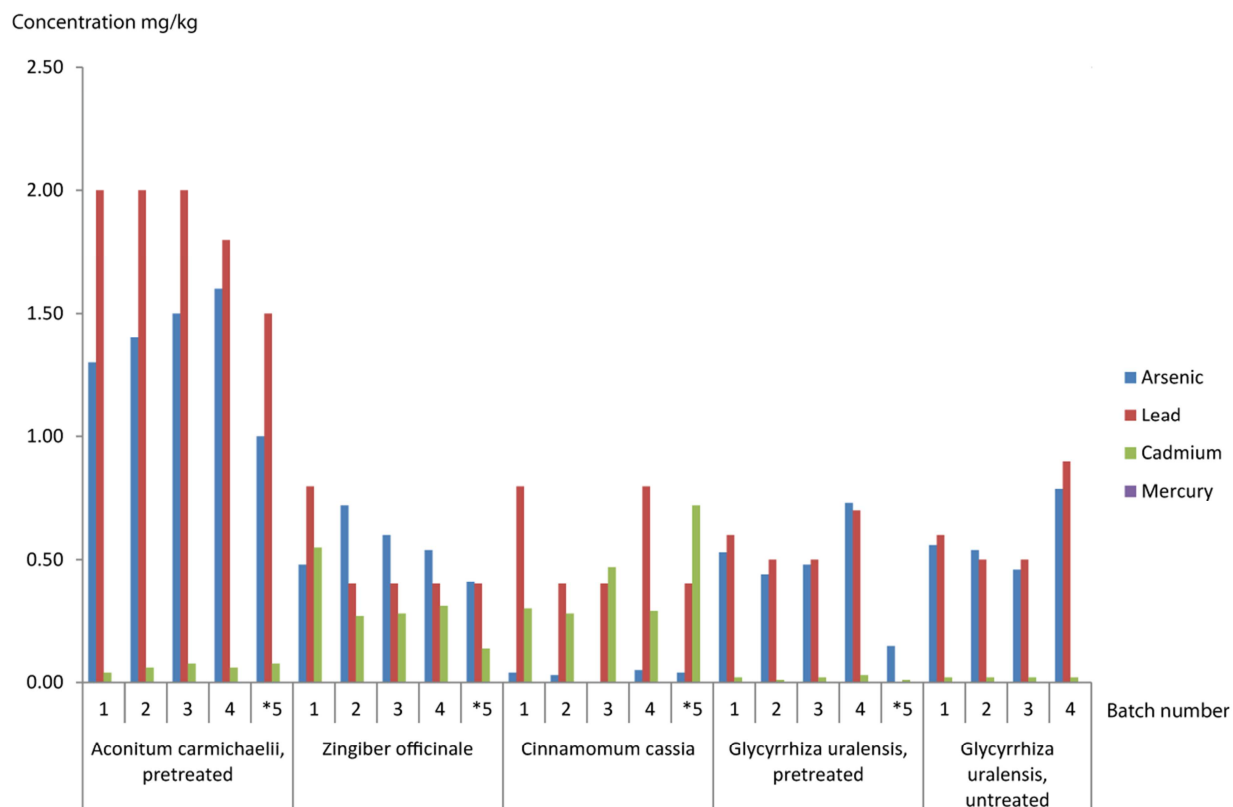


Figure 13. Concentration of arsenic, lead, cadmium and mercury analyzed in twenty-four batches of medicinal plants; * these batches were used for in vivo experiments

In all batches the ion-concentrations of Pb, Cd and Hg do not exceed the limits of 5.00 mg/kg Pb, 1.00 mg/kg Cd and 0.1 mg/kg Hg recommended for medicinal plants²⁵⁸.

5.1.1.1 Calculated intake of arsenic, lead, cadmium and mercury

The Joint Food and Agriculture Organization/World Health Organization Expert Committee on Food Additives established a provisional tolerable weekly intake (PTWI) of

- 15 µg/kg body weight for As (900 µg/person),
- 25 µg/kg body weight for Pb (1500 µg/person),
- 7 µg/kg body weight for Cd (420 µg/person) and
- 5 µg/kg body weight for Hg (300 µg/person)²⁵⁹.

Case example. The weekly heavy metal intakes were calculated according to an average daily dose of SNT decoction for a 60 kg person in China. For this calculation the highest amount of each heavy metal detected within one herbal group was taken and outlined in table 10. The LOQ of Hg (0.05 mg/kg) was used for the calculation.

Table 10. Composition of the highest amount of each heavy metal concentration detected within one herbal group for the calculation of the weekly intake of SNT decoction

Medicinal herb	Concentration mg/kg			
	Arsenic	Lead	Cadmium	Mercury
<i>A. carmichaelii</i> , pretreated	1.60	2.00	0.08	< 0.05
<i>Z. officinale</i>	0.72	0.80	0.55	< 0.05
<i>C. cassia</i>	0.05	0.80	0.72	< 0.05
<i>G. uralensis</i> , pretreated	0.73	0.70	0.03	< 0.05

Table 11. Case example: calculation of the weekly intake of SNT decoction, according to the average dose for a 60 kg person in China using the concentration shown in table 10

Medicinal herb	Concentration µg/person/week			
	Arsenic	Lead	Cadmium	Mercury
<i>A. carmichaelii</i> , pretreated	202.0	252.0	10.1	6.3
<i>Z. officinale</i>	45.4	50.4	34.7	3.2
<i>C. cassia</i>	1.1	16.8	15.1	1.1
<i>G. uralensis</i> , pretreated	122.6	117.6	5.0	8.4
Sum of weekly intake	371.1	436.8	64.9	19.0
PTWI for a 60 kg person	900.0	1500.0	420.0	300.0

This case example demonstrates that the consumption of each combination of the tested herbal batches for SNT would show As, Pb, Cd and Hg concentrations below the calculated sum of the provisional tolerable weekly intake (PTWI) (table 11).

5.1.2 The qualitative fingerprinting of SNT by HPLC

For the qualitative fingerprinting analysis of the SNT decoction (section 4.1.3.2) by HPLC successful separation was achieved using an aqueous buffer containing 15 mM *ortho*-phosphoric acid, 1.5 mM tetrabutylammoniumhydroxide and acetonitrile (pH 3), developed by

Reisch et al.²⁴⁶ in a gradient elution program (see table 4, section 4.1.3.3). The bioactive compounds of *G. uralensis* (liquiritin, isoliquiritin, liquiritigenin, isoliquiritigenin, licochalcon A and glycyrrhizic acid), *Z. officinale* (8-gingerol and 6-shogaol) and *C. cassia* (eugenol, cinnamaldehyde, 4-methoxy-cinnamaldehyde) were selected. The selection was based on compounds which were known to be isolated from the single herbs and which have shown bioactivity in previous studies^{103,117,179,222,260-265}. Most compounds in SNT showed strong UV absorbance at 240 nm, therefore this wavelength was used for the analysis of SNT fingerprint profile. The peaks in the SNT chromatogram were compared with the retention time (RT) and UV-spectra of the reference standards for the identification of single compounds in the SNT decoction.

In figure 14 the chromatogram of the fingerprint profile of SNT and the corresponding UV-spectra of the analyzed compounds are shown. Liquiritin was detected at RT 16.79 min, isoliquiritin at RT 25.74 min, eugenol at RT 29.06 min, liquiritigenin at RT 30.33 min, glycyrrhizic acid at RT 46.64 min. Liquiritin and glycyrrhizic acid were found to be the main components of the standards analyzed in this SNT decoction as well as the isoliquiritin and liquiritigenin compounds of *G. uralensis*. Eugenol is a bioactive component of *C. cassia*. No other compounds could be identified in this SNT fingerprint. The varying amounts of each herb in the decoction (ratio: 8:6:3:1) may have an influence on the concentration and therefore on the identification of compounds derived from each herb.

5 Results

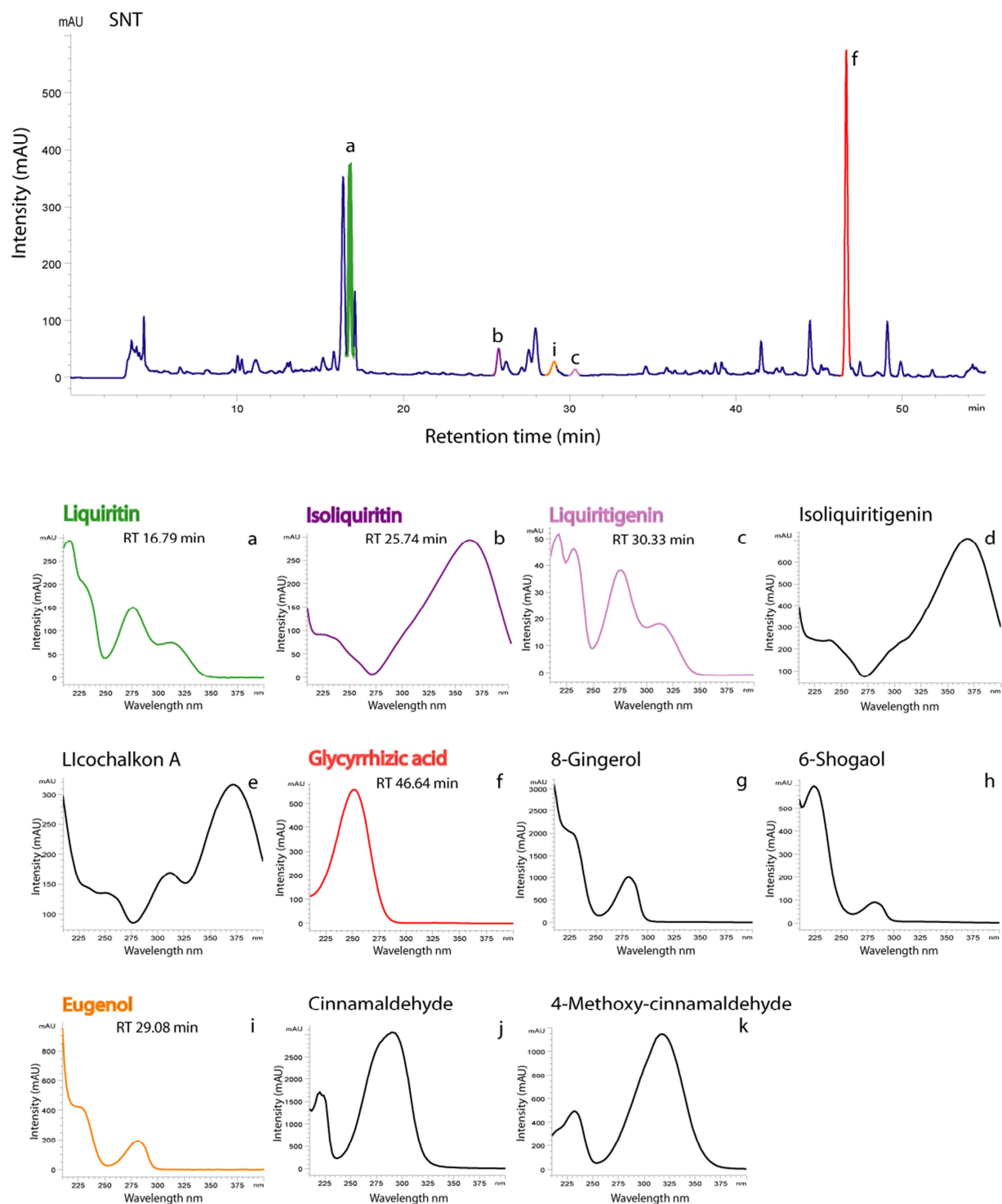


Figure 14. HPLC fingerprint profile of SNT; a-k = identified compounds of *G. uralensis* (liquiritin, isoliquiritin, liquiritigenin, isoliquiritigenin, licochalcon A and glycyrrhizic acid), *Z. officinale* (8-gingerol and 6-shogaol) and *C. cassia* (eugenol, cinnamaldehyde, 4-methoxy-cinnamaldehyde) and their corresponding UV-spectra; monitoring wavelength 240 nm

5.1.3 Identification and quantification of aconitine, hypaconitine and mesaconitine

The focus of this analysis is to find a simple and efficient method for the determination and quantification of the highly toxic aconitine-type alkaloids, aconitine, hypaconitine and mesaconitine in the roots of *A. carmichaelii*. It starts with the determination of the most effective extraction solvent which is followed by the analysis of the best HPLC condition associated with adequate eluents.

A precise peak separation was achieved by the optimization of a gradient system (table 3, section 4.1.3.3) using an aqueous buffer (A) containing 15 mM *ortho*-phosphoric acid and 1.5 mM tetrabutylammoniumhydroxide and acetonitrile (pH 3) (B), developed by Reisch et al.²⁴⁶. Established methods were applied in the examination of various batches of *A. carmichaelii* and the quantification of the diester diterpene alkaloids (DDAs), aconitine (AC), hypaconitine (HA) and mesaconitine (MA). Another point of interest was comparing the concentration of the DDAs obtained by extraction with organic solvents to the concentrations determined by the extraction of the decoction.

5.1.3.1 Optimization of the HPLC chromatographic conditions

The focus in establishing HPLC chromatographic conditions for the quantitative determination was influenced by the following requirements: adequate chemical information, a satisfying resolution of adjacent peaks, short time analysis and repeatability of the examination. A precise high-performance liquid chromatography method was developed for the quantitative determination of AC, HA and MA providing successful separation of the main toxic alkaloids in a short analysis time (see figure 15). This method therefore serves as the basis for reliable quantitative analysis. The wavelength used for the determination and quantification of AC, MA and HA was set at 233 nm, because this wavelength showed the maximum absorption of all the aconitines. The optimal separation of these alkaloids with similar chemical structures was achieved using a gradient system (see table 3, section 4.1.3.3) of an aqueous buffer containing 15 mM *ortho*-phosphoric acid and 1.5 mM tetrabutylammoniumhydroxide and acetonitrile (pH 3). This buffer was developed by Reisch et al.²⁴⁶.

5.1.3.2 Validation of the HPLC assay

For the quantification of AC, HA and MA by HPLC-DAD in *A. carmichaelii* roots, the peaks were compared with the RT and UV-spectra of the reference standards for their identification in the extracts. Linear regression analysis was performed using a calibration curve of ten concentrations. The regression equation for AC was $y = 4.4098x + 0.4458$ and the correlation coefficient (R^2) was 0.9992. For HA and MA the regression equations were $y = 7.9772x - 20.678$ ($R^2 = 0.9995$) and $y = 9.7577x - 16.899$ ($R^2 = 0.9991$), respectively (see table 12).

Table 12. Linear regression equation and linear range

Standard	Regression equation	R^2	Linear range ($\mu\text{g/mL}$)
Aconitine	$y = 4.4098x + 0.4458$	0.9992	2.00 – 160.00
Hypaconitine	$y = 7.9772x - 20.678$	0.9995	2.88 – 230.00
Mesaconitine	$y = 9.7577x - 16.899$	0.9991	1.88 – 150.00

x stands for the alkaloid concentration in $\mu\text{g/mL}$, y denotes the area under the curve, R^2 is the correlation coefficient of the equation

The repeatability and recovery results are presented in table 13 and the intra-day and inter-day precision are shown in table 14. The contents of AC and MA were below the LOD in all batches, therefore only HA could be taken into consideration for repeatability and precision tests. The LOD and the LOQ were determined to be 0.3 $\mu\text{g/mL}$ and 1.01 $\mu\text{g/mL}$, respectively for AC, HA and MA.

Table 13. Repeatability of the developed HPLC method for the determination of HA extracted with MeOH:H₂O (1:1) and recovery of AC, HA and MA in an unprocessed *A. carmichaelii* sample extracted with MeOH (batch FZ-09 – see table 15) by adding 15.5 μg AC, 28.0 μg MA and 102.0 μg HA

Compound	Initial amount ^a (μg)	Added amount (μg)	Detected amount ^b (μg)	Recovery (%)	RSD* (%)
Aconitine	--	15.5	15.6 $\pm$ 0.19	100.9	1.20
Mesaconitine	--	28.0	27.7 $\pm$ 0.34	98.9	0.69
Hypaconitine	200.8 $\pm$ 2.31	102.0	301.7 $\pm$ 2.09	98.8	1.22

^a Repeatability; mean $\pm$ SD (n = 5); AC and MA could not be detected (< LOD)

^b Mean $\pm$ SD (n = 5)

* relative standard deviation

Table 14. Intra-day and inter-day precision of the developed HPLC method for the determination of HA in an unprocessed Aconitum sample extracted with MeOH:H₂O (1:1) (batch FZ-09 – see table 15)

Parameter	Peak area ^a	RSD (%) [*]
Intra-day ^b	134.1 ± 0.79	0.59
Inter-day ^c	133.5 ± 0.75	0.56

^a Mean ± SD

^b Samples analyzed five times during one day (n = 5)

^c Samples analyzed twice a day over three consecutive days (n = 6)

^{*} relative standard deviation

The HPLC-DAD analysis showed good repeatability, precision and recovery and thus provides a reliable basis for the quantification of the DDAs in extracts of *A. carmichaelii*. The developed HPLC method is time efficient (23 min) which allows a high throughput of *Aconitum* extracts in a relatively short time compared to that of other investigators^{69,247,266-268}.

5.1.3.3 Optimization of the extraction conditions and quantitative analysis

Extraction of the powdered processed and unprocessed *A. carmichaelii* roots was performed using the method of Lu et al.⁶⁹. The amount of 0.5 mg ground root of *A. carmichaelii* was extracted in 5 mL of the following organic solvents respectively:

- methanol
- methanol-water 1:1
- water
- 1 % aqueous hydrochloric acid.

In this way it was possible to determine the most effective extraction solvent for the quantification of AC, HA and MA in various batches. The average value of the DDAs in each sample was calculated and expressed as their amount in a specific solid sample. The results were compared between all the extraction agents (see table 15).

Table 15. Content of AC, HA and MA in eight processed and one unprocessed *A. carmichaelii* batches

		AC (μg/g)		HA (μg/g)		MA (μg/g)	
Sample (batch number)	solvent	Mean ±SD	RSD	Mean ± SD	RSD	Mean ±SD	RSD
processed Fu Zi root							
FZ-01-06	MeOH	nd	nd	88.80±0.79	0.89	nd	nd
	MeOH:H ₂ O	nd	nd	nd	nd	nd	nd
	H ₂ O	nd	nd	nd	nd	nd	nd
	1% HCl	nd	nd	nd	nd	nd	nd
FZ-02-09	MeOH	nd	nd	nd	nd	nd	nd
	MeOH:H ₂ O	nd	nd	nd	nd	nd	nd
	H ₂ O	nd	nd	nd	nd	nd	nd
	1% HCl	nd	nd	nd	nd	nd	nd
FZ-03-10	MeOH	nd	nd	nd	nd	nd	nd
	MeOH:H ₂ O	nd	nd	nd	nd	nd	nd
	H ₂ O	nd	nd	nd	nd	nd	nd
	1% HCl	nd	nd	nd	nd	nd	nd
FZ-04-10	MeOH	nd	nd	nd	nd	nd	nd
	MeOH:H ₂ O	nd	nd	nd	nd	nd	nd
	H ₂ O	nd	nd	nd	nd	nd	nd
	1% HCl	nd	nd	nd	nd	nd	nd
FZ-05-10	MeOH	nd	nd	nd	nd	nd	nd
	MeOH:H ₂ O	nd	nd	nd	nd	nd	nd
	H ₂ O	nd	nd	nd	nd	nd	nd
	1% HCl	nd	nd	nd	nd	nd	nd
FZ-06-10	MeOH	nd	nd	nd	nd	nd	nd
	MeOH:H ₂ O	nd	nd	nd	nd	nd	nd
	H ₂ O	nd	nd	nd	nd	nd	nd
	1% HCl	nd	nd	nd	nd	nd	nd
FZ-07-10	MeOH	nd	nd	nd	nd	nd	nd
	MeOH:H ₂ O	nd	nd	nd	nd	nd	nd
	H ₂ O	nd	nd	nd	nd	nd	nd
	1% HCl	nd	nd	nd	nd	nd	nd
FZ-08-10-BJ	MeOH	nd	nd	72.47±0.33	0.46	nd	nd
	MeOH:H ₂ O	nd	nd	nd	nd	nd	nd
	H ₂ O	nd	nd	nd	nd	nd	nd
	1% HCl	nd	nd	nd	nd	nd	nd
unprocessed Fu Zi root							
FZ-09	MeOH	nd	nd	269.34±0.58	0.22	nd	nd
	MeOH:H ₂ O	nd	nd	199.48±0.76	0.38	nd	nd
	H ₂ O	nd	nd	71.32±0.84	1.18	nd	nd
	1% HCl	nd	nd	251.12±0.49	0.20	nd	nd

Mean ± SD (n = 3); nd = not detectable (< LOD)

In general only HA could be detected, AC and MA were below the LOD (0.3 µg/mL) in all batches. The most effective extraction was carried out using MeOH (269.34 µg/g HA), followed by 1 % hydrochloric acid (251.12 µg/g HA) and MeOH:H₂O (199.48 µg/g HA) (see table 15, FZ-09). Two batches in eight of processed *A. carmichaelii* from China showed HA contents: batch FZ-01-06 (88.80 µg/g) and batch FZ-08-10-BJ (72.47 µg/g).

To summarize, the HPLC-DAD analysis developed showed good repeatability, precision and recovery and therefore can serve as a simple method for the quantification of the DDAs in *Aconitum* tubers and roots. In two of eight processed batches of *A. carmichaelii* extracted with MeOH, HA content was found, whereas in the extraction using the other organic solvents (MeOH:H₂O, H₂O, 1 % HCl) none of the aconitines could be determined. In the unprocessed batch of *A. carmichaelii*, concentrations of HA – varying from 71.32 µg/g (extraction with H₂O) to 269.34 µg/g (extraction with MeOH) were detected. Therefore MeOH was defined as the most effective extraction solvent in this study.

5.1.4 Comparison of hypaconitine concentrations in aqueous decoction and methanolic extraction

The focus of this project was to keep the analysis as close as possible to the application conditions, therefore decoctions (procedure described in section 4.1.3.2) of the same batches used for extraction with organic solvent were prepared, lyophilized and analyzed by HPLC. In addition, a SNT decoction was prepared using unprocessed *A. carmichaelii* (batch FZ-09) and analyzed on equal terms.

Table 16. Concentration of HA obtained by the methanolic extraction compared to that determined by the aqueous extraction (decoction) of processed and unprocessed *A. carmichaelii* roots and the decoction of SNT, containing the same batch of unprocessed root of *A. carmichaelii*

Sample (batch number)	MeOH		Decoction		Decoction	
	HA (µg/g)	RSD	HA (µg/100 mg lyo)	RSD	HA (µg/mL decoction)	RSD
processed						
<i>A. carmichaelii</i> root						
FZ-01-06	88.80 ± 0.79	0.89	nd	nd	--	--
FZ-08-10-BJ	72.47 ± 0.33	0.46	nd	nd	--	--
unprocessed						
<i>A. carmichaelii</i> root						
FZ-09	269.34 ± 0.58	0.22	62.10 ± 1.05	1.68	5.22 ± 0.09	1.68
SNT with unprocessed						
<i>A. carmichaelii</i> root						
SNT with FZ-09	--	--	nd	nd	--	--

Mean ± SD (n = 3); nd = not detectable (< LOD), (--) not measured, lyo = lyophilisate

In table 16 the batches (FZ-01-06, FZ-08-10-BJ, FZ-09), in which HA could be detected by means of methanolic extraction, are listed and compared with the results obtained from the analysis of the decoction. In the decoctions of the eight processed *A. carmichaelii* batches AC, HA and MA was not detected, even in batches FZ-01-06 and FZ-08-10-BJ, HA was not detected (see table 16). In the decoction with unprocessed *A. carmichaelii* root (batch FZ-09) a concentration of 62.1 ± 1.05 µg/100 mg lyophilisate was determined, which is less than one fourth of the amount detected in the extraction with MeOH. The concentration of HA in the decoction (µg/mL) was calculated to make the results comparable. These values should be considered as an approximation.

Calculation: e.g. batch FZ-09 (unprocessed *A. carmichaelii*)

An amount of 100 mg/mL lyophilisate of decoction were analyzed, 50 mL decoction produce averaged 420 mg lyophilisate and 400 mL decoction result from one decoction procedure. This result in 3360 mg lyophilisate per 400 mL decoction, therefore a simple calculation leads to the approximate amount of 5.22 µg HA in 1 mL decoction:

$$\begin{aligned}
 &100 \text{ mg} \dots\dots 62.1\mu\text{g} \\
 &\underline{3360 \text{ mg} \dots\dots} \times \mu\text{g} \\
 &= 2086.6 \mu\text{g}/400 \text{ mL} = \underline{5.22 \mu\text{g/mL}}
 \end{aligned}$$

Figure 15 presents the standard solution (containing AC, HA and MA) as control and the chromatogram of the decoction with unprocessed and processed *A. carmichaelii*. The UV-spectra correspond to the colored peaks and are additionally marked with characters (a-i). In the chromatogram of unprocessed *A. carmichaelii* (II), the UV-spectra (b, c, e) of the peaks with the same RT as MA and AC alter slightly but significantly to that of AC and MA (I, a) which is the same in all three chemical structures, whereas peak d is detected as HA due to a RT (17.5 min) and a UV-spectra corresponding to that of HA (I, a). In the chromatogram of processed *A. carmichaelii* the UV-spectra (III, f – i) differ completely from that of AC, HA and MA.

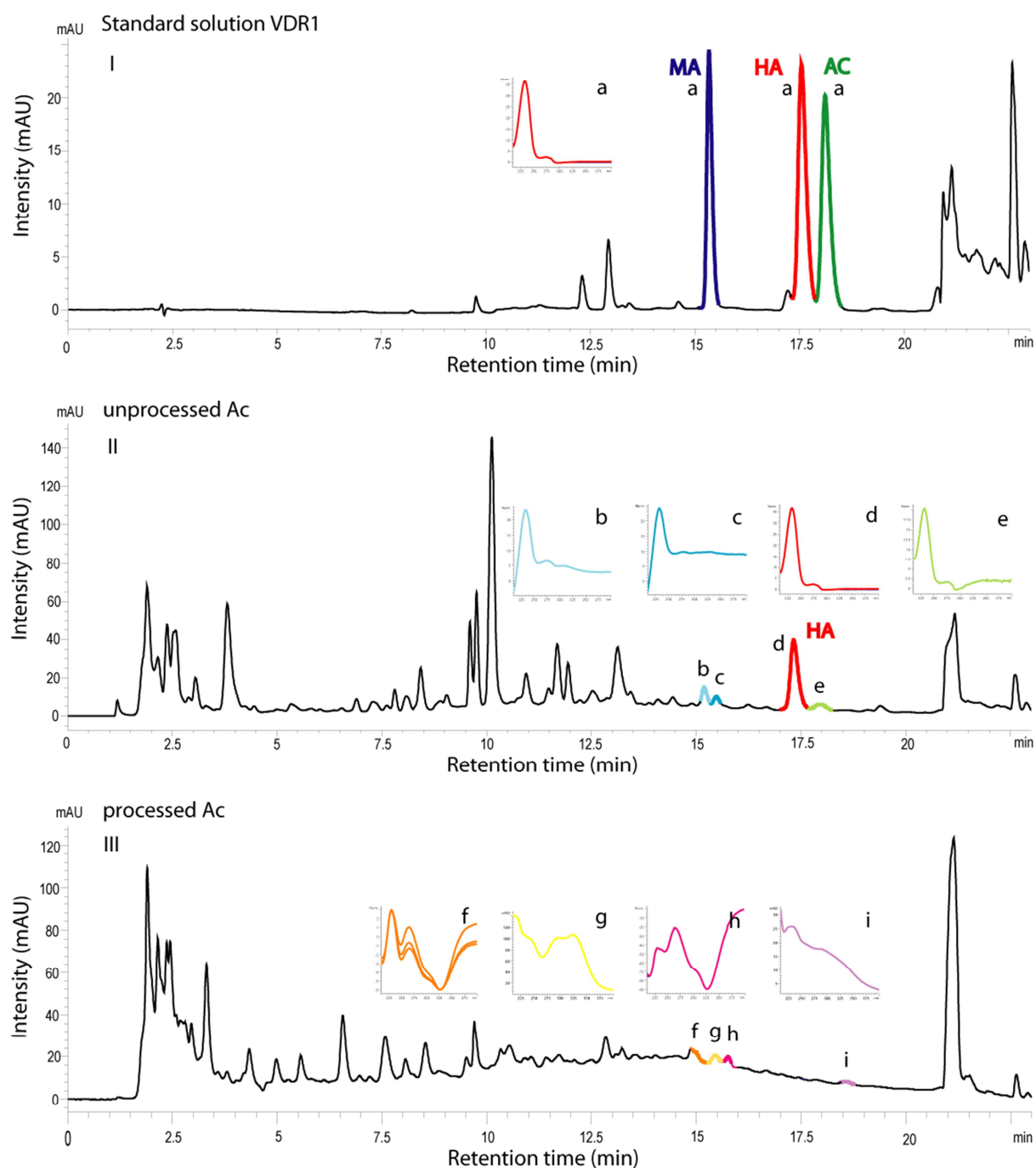


Figure 15. HPLC profile of standard solution VDR1 (I), decoction of unprocessed *A. carmichaelii* (II) and processed *A. carmichaelii* (III); a-i = corresponding UV-spectra to colored peaks; a = UV-spectra of AC, HA and MA; monitoring wavelength 233 nm

In addition, the result of the analysis of SNT decoction containing unprocessed *A. carmichaelii* (FZ-09) is shown in table 16. Surprisingly, HA could not be detected. In figure 16 the HPLC profile of SNT decoction with unprocessed *A. carmichaelii* (II) is shown. The UV-spectra (b-f) corresponding to the colored peaks at the same RT as AC, HA and MA differ completely to that of the aconitines (I, a).

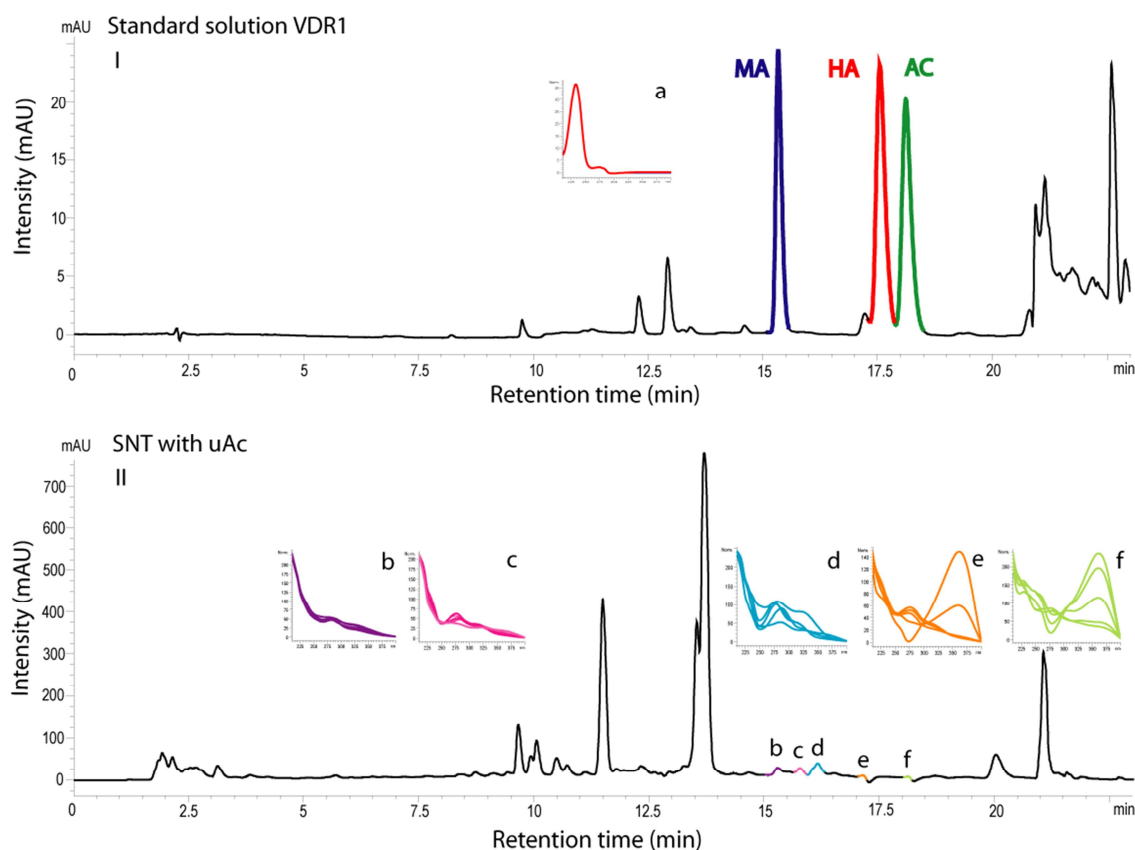


Figure 16. HPLC profile of standard solution VDR1 (I) and HPLC fingerprint profile of SNT decoction with unprocessed *A. carmichaelii* (II); a-f = corresponding UV-spectra to colored peaks; a = UV-spectra of AC, HA and MA; monitoring wavelength 233 nm

To summarize, none of the aconitines could be detected either in the decoction of processed *A. carmichaelii* or in the SNT decoction containing unprocessed *A. carmichaelii*. Whereas HA was detected in the decoction with unprocessed *A. carmichaelii*.

These results provoke some interesting questions: Why could we detect $62.1 \pm 1.05 \mu\text{g}$ HA in 100 mg lyophilisate with unprocessed *A. carmichaelii* root, whereas in the lyophilisate of SNT

containing unprocessed *A. carmichaelii* roots from the same batch and in the same concentration no HA was determined? What happened to the HA in the whole formula?

It is also important to stress that these results refer only to the batches used in this project and cannot be transferred to other batches 1:1, since the heavy metal contamination and compounding are dependent on the various factors described in section 1.3.

5.1.5 Analysis of herbal interactions

Taking the results obtained by the analysis of SNT decoction with unprocessed *A. carmichaelii* (section 5.1.4) various questions arise regarding possible chemical interaction within the compounds of the herbs used for the decoction of SNT. This is the focus of the herbal interaction analysis, in which the amount and concentration of single herbs in the decoctions are constant and equal to those of the SNT decoction. The only varying factor is the combination of the single herbs for the decoction process. A point of interest is analyzing the concentrations of the aconitines, in particular HA, in the decoctions with different herbal combinations in the order to discover which herb(s) and, additionally, which compound(s) of herb(s), is/are responsible for the chemical interaction with AC, HA and MA.

Table 17. Combinations of the herbs for the decoction to analyze possible herbal interactions

Herb ^a	+	Herb	+	Herb	Abbreviation	Ratio ^b
unprocessed <i>A. carmichaelii</i>		--		--	uAc	6
unprocessed <i>A. carmichaelii</i>		<i>G. uralensis</i>		--	uAc/Gu	6:8
unprocessed <i>A. carmichaelii</i>		<i>C. cassia</i>		--	uAc/Cc	6:1
unprocessed <i>A. carmichaelii</i>		<i>Z. officinale</i>		--	uAc/Zo	6:3
unprocessed <i>A. carmichaelii</i>		<i>C. cassia</i>		<i>Z. officinale</i>	uAc/Cc/Zo	6:1:3

^a batch FZ-09 of *A. carmichaelii*

^b same ratio as in SNT

In table 17 the combination of the herbs, their abbreviations and ratio are shown. The results of the concentration of HA in the decoctions are shown in table 18. For the explanation of the calculation of HA µg per mL decoction see 5.1.4.

Table 18. Concentration of HA in single unprocessed *A. carmichaelii* decoction compared to that of herbal combinations

Herbal combination for decoction	HA ($\mu\text{g}/100\text{ mg lyophilisate}$)		HA $\mu\text{g}/\text{mL}$ decoction	
	Mean $\pm$ SD	RSD	Mean $\pm$ SD	RSD
uAc	62.10 ± 1.05	1.68	5.22 ± 0.09	1.68
uAc/Gu	nd	--	--	--
uAc/Cc	46.95 ± 0.48	1.02	1.64 ± 0.02	1.02
uAc/Zo	55.60 ± 1.01	1.82	2.00 ± 0.04	1.82
uAc/Cc/Zo	34.25 ± 2.14	6.26	1.23 ± 0.08	6.26

For abbreviations and ratio see table 17; uAc batch = FZ-09; same herbal concentration in all decoctions; nd = not detectable ($< \text{LOD}$); (--) not measured

The analysis of the decoction of herbal combinations led to the following results: Interestingly, in the combination of unprocessed *A. carmichaelii* with *G. uralensis* (ratio of 6:8) no aconitines could be determined. However the concentration of unprocessed *A. carmichaelii* with *C. cassia* (ratio of 6:1) led to the detection of $46.95 \pm 0.48\text{ }\mu\text{g}$ HA per 100 mg lyophilisate and furthermore resulted in a concentration of $1.64 \pm 0.02\text{ }\mu\text{g}$ HA per mL decoction. The combination of unprocessed *A. carmichaelii* with *Z. officinale* (ratio of 6:3) resulted in $55.60 \pm 1.01\text{ }\mu\text{g}$ HA per 100 mg lyophilisate and $2.00 \pm 0.04\text{ }\mu\text{g}$ HA per mL decoction. In the combination of unprocessed *A. carmichaelii* with *C. cassia* and *Z. officinale* (ratio of 6:1:3) the amount of $34.25 \pm 2.14\text{ }\mu\text{g}$ HA per 100 mg lyophilisate was determined and a concentration of $1.23 \pm 0.08\text{ }\mu\text{g}$ HA per mL decoction calculated. Compared to the content of HA in single unprocessed *A. carmichaelii* decoction, which was further calculated as $5.22 \pm 0.09\text{ }\mu\text{g}$ per mL decoction, the concentration of HA in the combination of unprocessed *A. carmichaelii* and *C. cassia* was calculated to be less than one third (31.4 %), that of unprocessed *A. carmichaelii* and *Z. officinale* was 38.3 % and the combination of both, *Z. officinale* and *C. cassia*, with unprocessed *A. carmichaelii* even resulted in an even lower concentration of HA which was less than one fourth (23.6 %).

These discoveries lead to the conclusion that *G. uralensis* had the most influence on the concentration of HA in the combination with unprocessed *A. carmichaelii* using the same ratio as in SNT decoction, but also *C. cassia* and *Z. officinale* showed a significant reduction of HA per mL decoction.

5.1.5.1 Analysis of the interaction between the aconitines with single compounds of *Z. officinale*, *C. cassia* and *G. uralensis*

The objective of this analysis was to get further information about the detoxification of *A. carmichaelii*, more precisely about the ability of various bioactive compounds of *Z. officinale* (8-gingerol and 6-shogaol), *C. cassia* (eugenol, cinnamaldehyde and 4-methoxycinnamaldehyde) and *G. uralensis* (liquiritin, isoliquiritin, liquiritigenin, isoliquiritigenin, licochalcon A and glycyrrhizic acid) to interact with AC, HA and MA. As previously mentioned in section 5.1.3, the selection of these constituents was based on previous studies indicating a bioactivity of each single compound. For these investigations 100 μL of each reference standard (1 mg/mL) were each gently mixed with 15 μL of standard solution VDR1 (52 μg AC, 50.7 μg HA, 44.7 μg /mL MA) prior to the injection into the HPLC system.

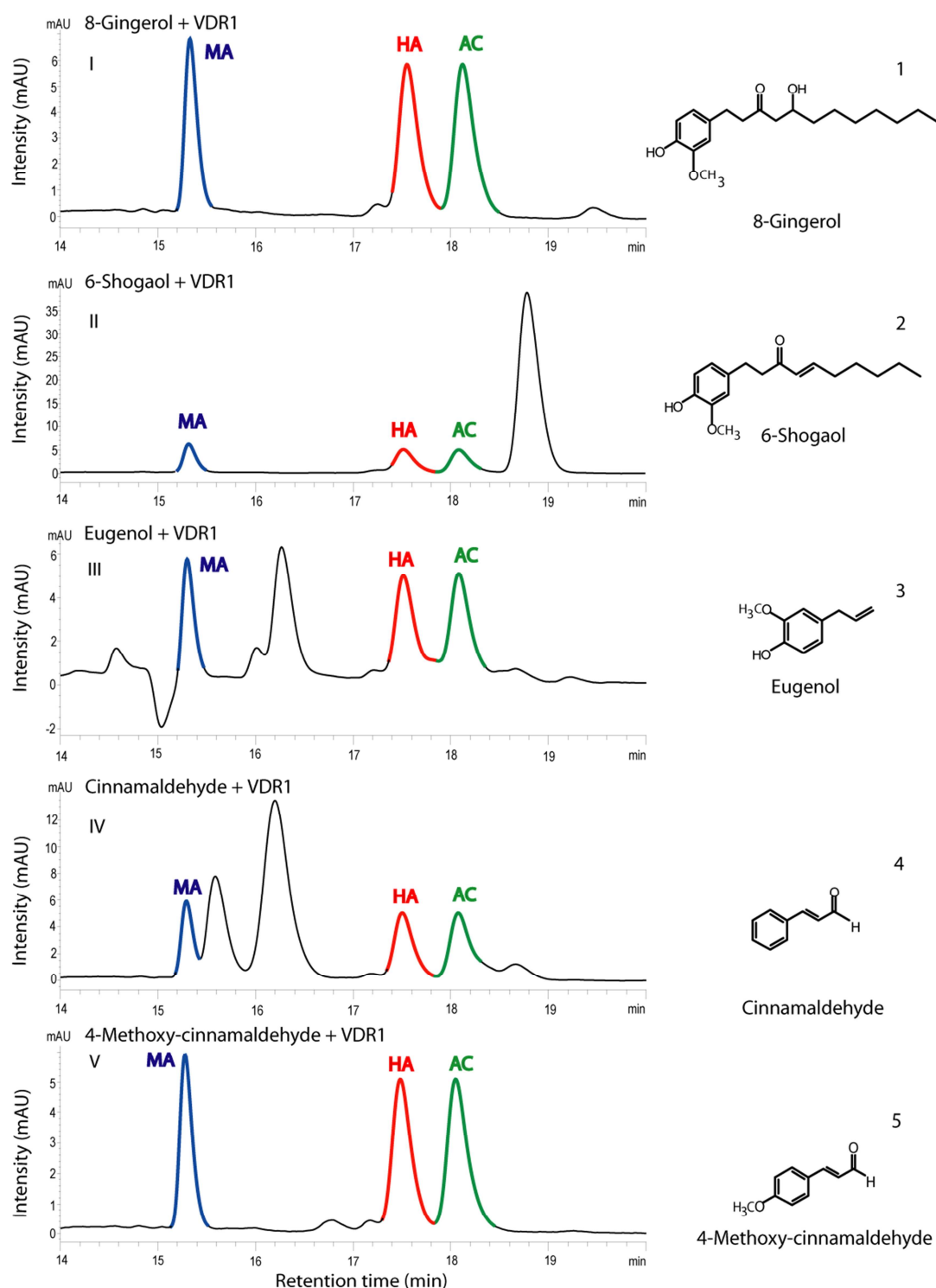


Figure 17. Section of HPLC profiles of the mixtures: standard solution VDR1 with bioactive compounds of *Z. officinale* (8- gingerol (I), 6-shogaol (II)) and *C. cassia* (eugenol (III), cinnamaldehyde (IV), 4-methoxy-cinnamaldehyde (V)); 1-5 = chemical structure of bioactive compounds of *Z. officinale* und *C. cassia*; monitoring wavelength 233 nm

Figures 17 and 18 demonstrate the outcome of these experiments. In the mixtures 8-gingerol, 6-shogaol, eugenol, cinnamaldehyde, 4-methoxy-cinnamaldehyde with standard solution VDR1, there were no changes observed in the amount of free AC, HA and MA (figure 17). Figure 18 shows the analysis of the interaction between bioactive compounds of *G. uralensis* and standard solution VDR1. The concentration of AC, HA and MA remained constant in the mixture of liquiritigenin, isoliquiritigenin, licochalcon A and glycyrrhizic acid with VDR1 as compared to that of solo VDR1. In contrast, in the mixture of isoliquiritin with VDR1 free AC, HA and MA were undetectable (see aberration of the UV-spectra) and in the mixture of liquiritin with VDR1 the amount of free MA and AC were reduced and free HA could not be determined. The UV-spectra of the correspondent colored peaks show a significant difference in comparison to the UV-spectra of AC, HA and MA. From these observations it can be assumed that liquiritin and isoliquiritin of *G. uralensis* interact with AC, HA and MA whereas all other tested compounds have no influence on the concentration of AC, HA and MA.

5 Results

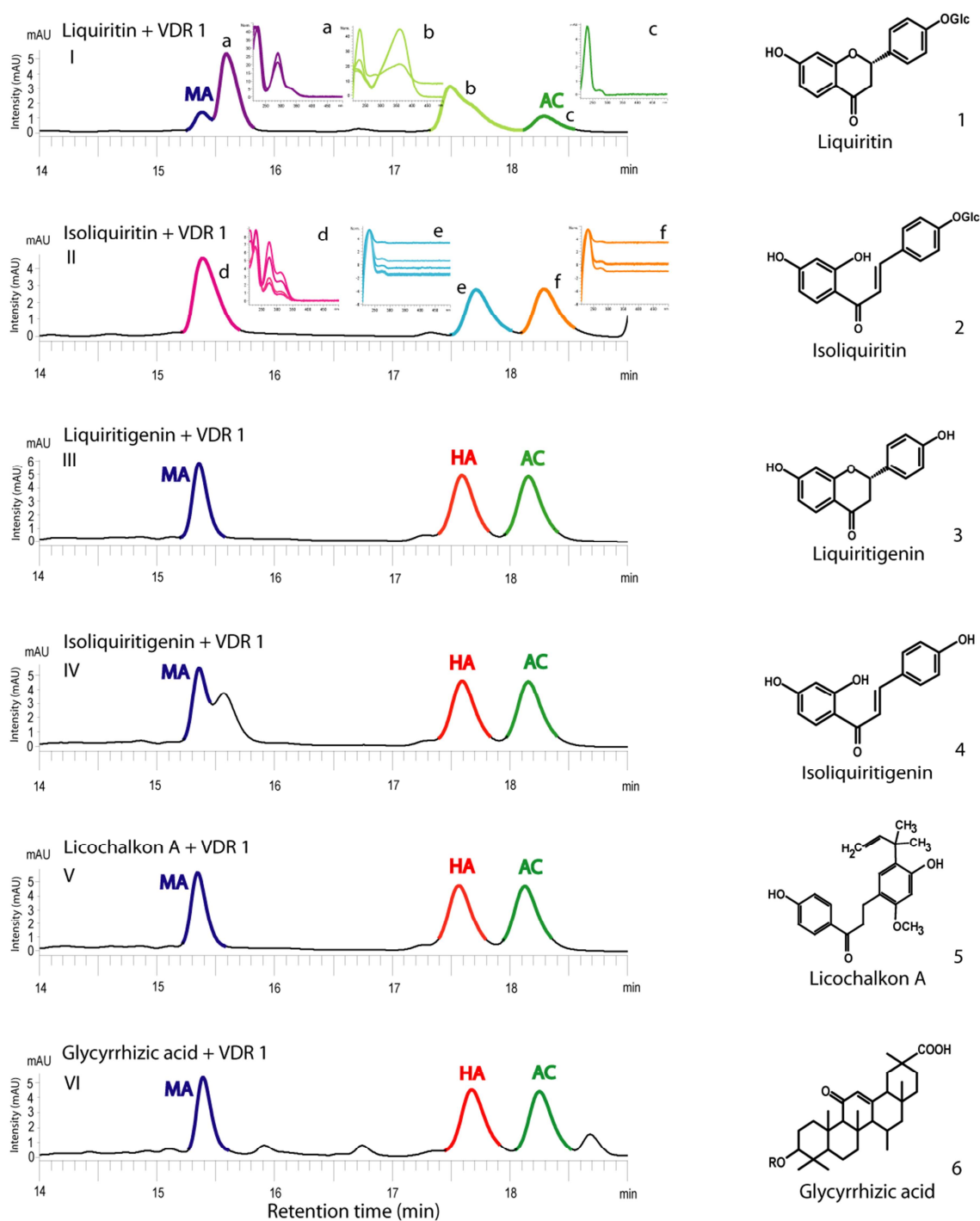


Figure 18. Section of HPLC profile of the combination of standard solution VDR1 with bioactive compounds of *G. uralensis* (liquiritin (I), isoliquiritin (II), liquiritigenin (III), isoliquiritigenin (IV), licochalcon A (V) and glycyrrhizic acid (VI)); 1-6 = chemical structure of bioactive compounds of *G. uralensis* ((6) R = β -glcA²- β -glcA); a-f = corresponding UV-spectra to colored peaks; c = UV-spectra of AC, HA and MA; monitoring wavelength 233 nm

These results demonstrate an ability of liquiritin and isoliquiritin to interact with AC, HA and MA and lead to further questions about the respective concentration and ratio which are necessary to detect a reduction of free AC, HA and MA. It is a further interesting aspect to find out in which way the chemical interaction between isoliquiritin, liquiritin and the aconitines occurs. Which effect is responsible for this behavior?

*5.1.5.2 Analysis of the interaction between the aconitines with isoliquiritin (a single compound of *G. uralensis*)*

The following experiments focus on the interaction between isoliquiritin and the aconitines, AC, HA and MA, more precisely, on the concentration of isoliquiritin that is necessary to reduce the aconitines. Figure 19 shows the outcome of mixing isoliquiritin in various concentrations with standard solution VDR1. It was discovered that with the amount of 20 µg of isoliquiritin the VDR1 content, consisting of 0.78 µg AC, 0.76 µg HA and 0.67 µg MA per 15 µL (2.21 µg aconitines in total), is reduced but still detectable, whereas in the presence of 30 µg isoliquiritin, free AC, HA and MA could not be detected. These findings lead to the assumption that at least the 10-fold quantity of isoliquiritin is required to reduce the aconitines to an undetectable level.

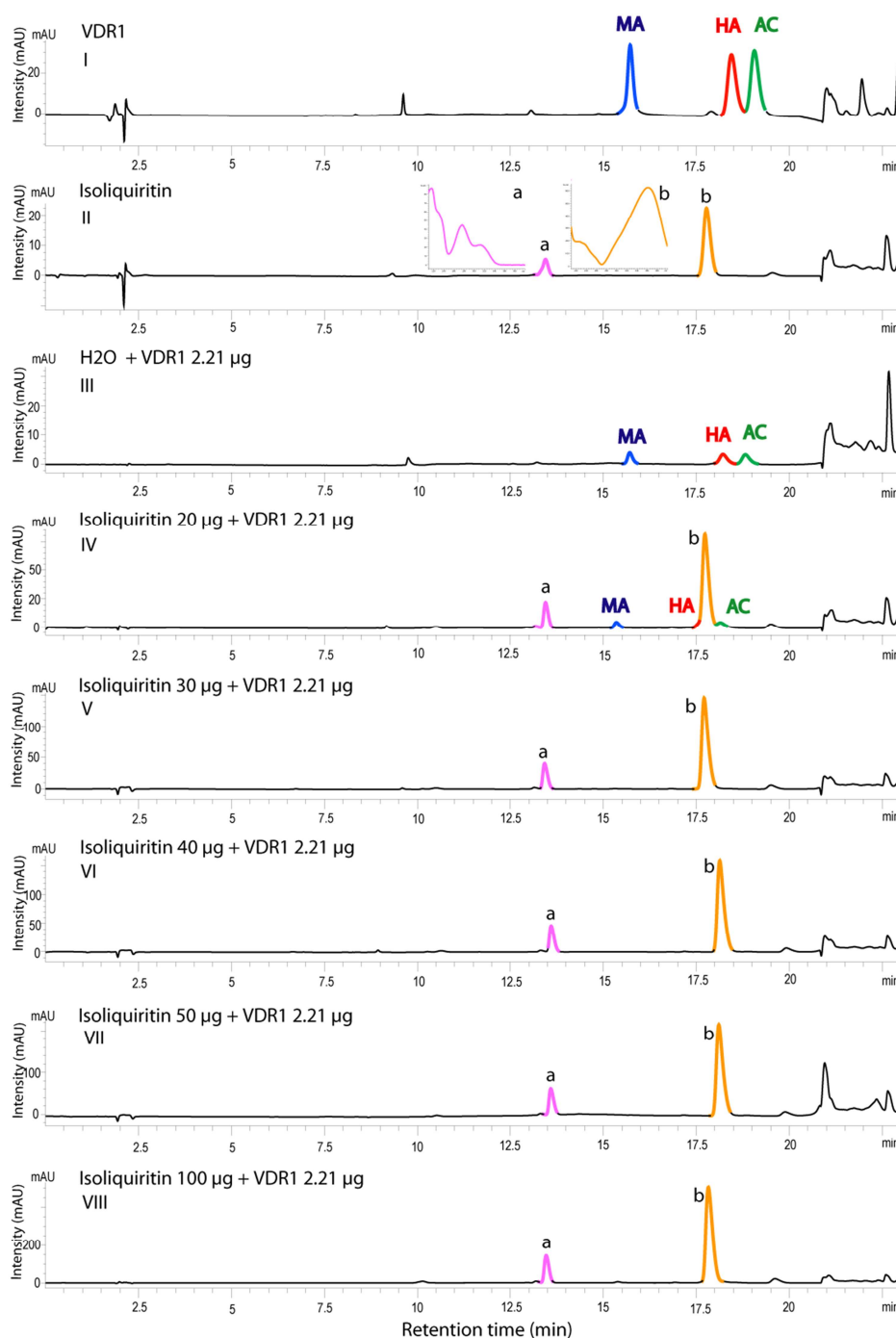


Figure 19. Potency of isoliquiritin to reduce the amount of AC, HA and MA; standard solution VDR1 (I), isoliquiritin (II), deionized water with 15 µL VDR1 (III); I, II and III served as control, IV – VIII = isoliquiritin (20, 30, 40, 50, 100 µL stock solution 1 mg/mL) with 15 µL standard solution VDR (2.21 µg aconitines in total) filled up with deionized water to 115 µL; a = corresponding UV-spectra to colored peak representing liquiritin (existed in a small amount in the reference standard of isoliquiritin); b = corresponding UV-spectra to colored peak representing isoliquiritin; monitoring wavelength 233 nm

An interesting observation was made, shown in figure 20, while questioning, what had happened to AC, HA and MA in the mixture with isoliquiritin. In the last part of the peak of isoliquiritin in the mixture with VDR1, an altered UV-spectrum appeared. Its shape resembles a mixture of the UV-spectra of free AC, HA and MA and that of free isoliquiritin with the same absorption maxima of the aconitines at 233 nm, as well as that of isoliquiritin at 362 nm (figure 21).

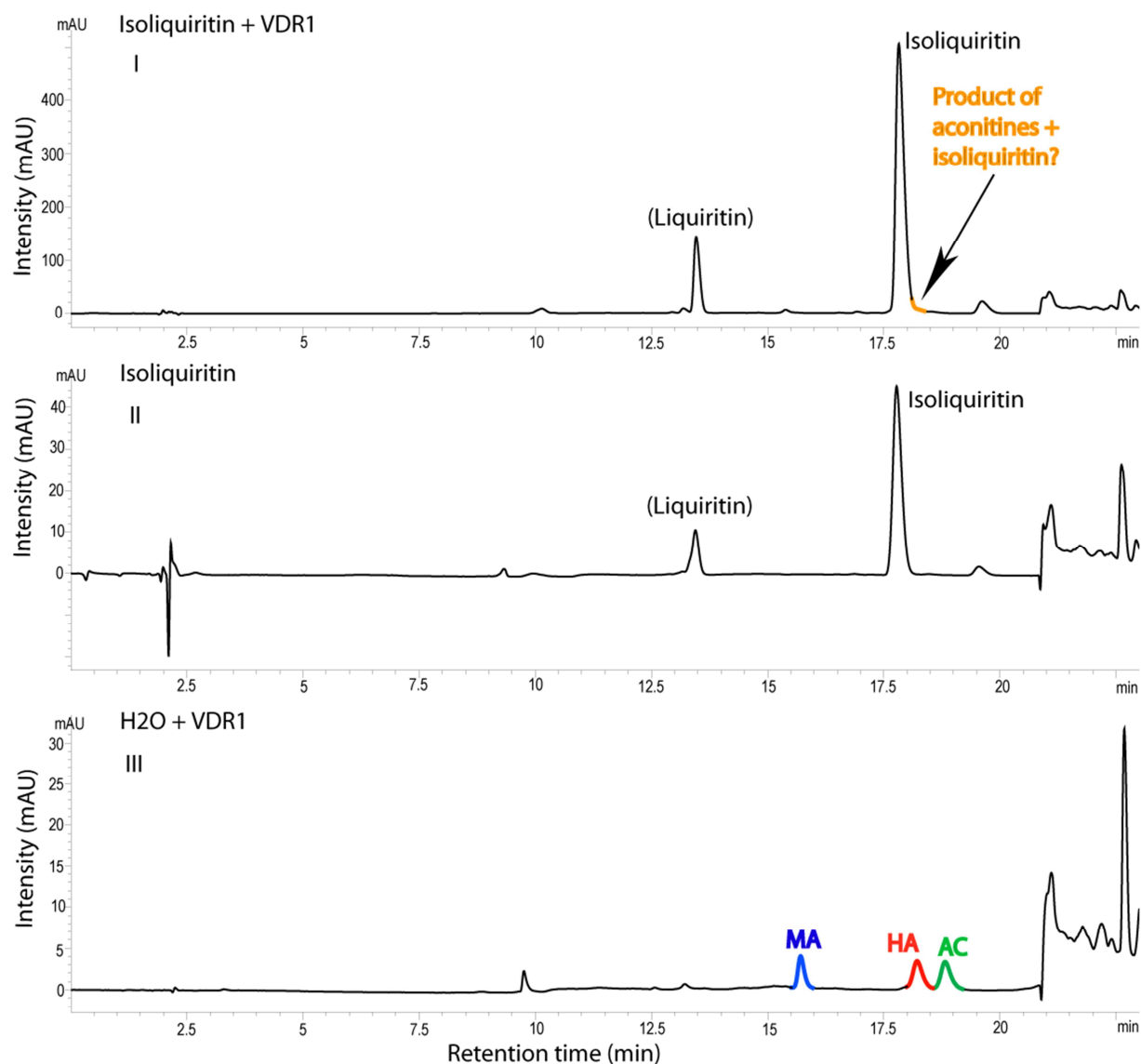


Figure 20. What happens to MA, HA and AC in the mixture with isoliquiritin? I isoliquiritin (100 μ L stock solution 1 mg/mL) with 15 μ L standard solution VDR1 (liquiritin existed in a small amount in the reference standard of isoliquiritin), II isoliquiritin, III deionized water with VDR1, II and III served as control

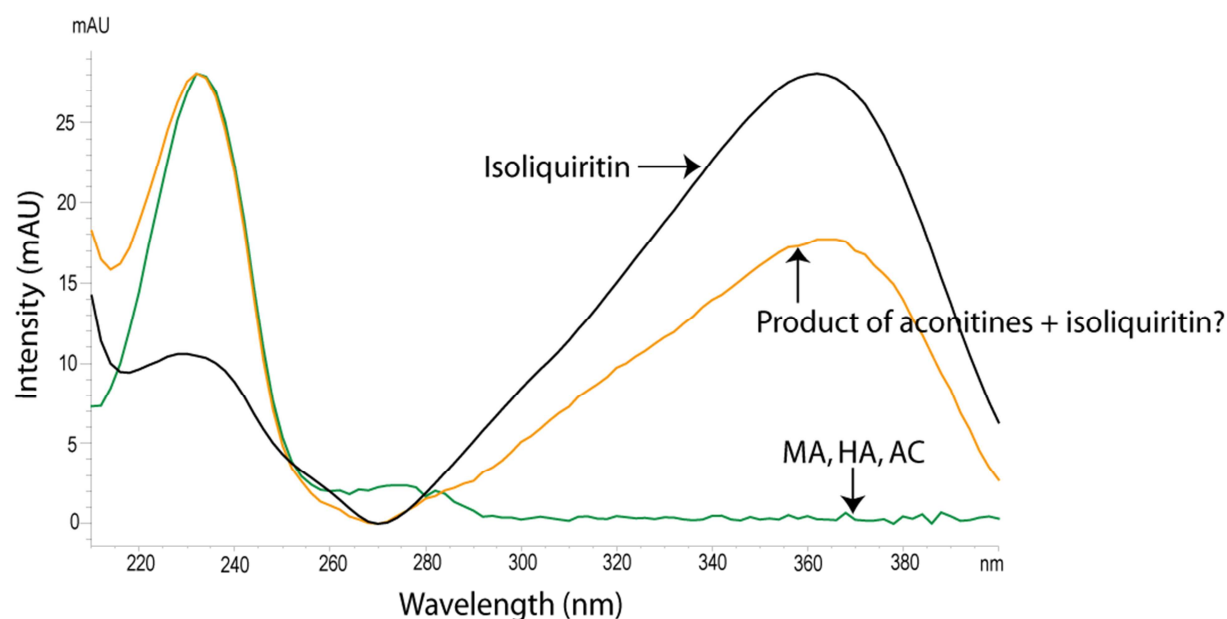


Figure 21. Corresponding UV-spectra of figure 20 of AC, HA and MA, isoliquiritin and the product of the aconitines with isoliquiritin; monitoring wavelength 233 nm

As shown in figure 21 the complex formed had the same maximum and intensity at 233 nm as free aconitine. However, the small maximum of aconitine at 274 nm had been lost since the complex formed showed a similar minimum at this wavelength to the free isoliquiritin. At 362 nm the new complex indicated a maximum equivalent to free isoliquiritin whereas the signal showed less intensity.

5.1.6 Analysis of the complex of aconitine and liquiritin

NMR spectroscopy is a useful method for analyzing the structure and the stability of supramolecular complexes. Preliminary results obtained by the analysis of 1:1 mixtures of liquiritin, isoliquiritin, liquiritigenin and isoliquiritigenin with aconitine demonstrated chemical shift changes of selected protons in all spectra. Liquiritin and liquiritigenin showed the largest shift changes and due to the main occurrence of liquiritin in *G. uralensis*, the decision was made to continue the subsequent experiments with liquiritin. Due to the poor solubility of aconitine and liquiritin in water, it was not possible to perform these experiments in water. By means of solubility tests, it was discovered that 1/3 of MeOD in D₂O was necessary to dissolve the

concentration of aconitine used and 2/3 of MeOD in D₂O was used as the concentration of liquiritin was increased for the analysis of the binding constants. ¹H-NMR spectra of aconitine and liquiritin are shown in figures 22, 23 and tables 19, 20, respectively.

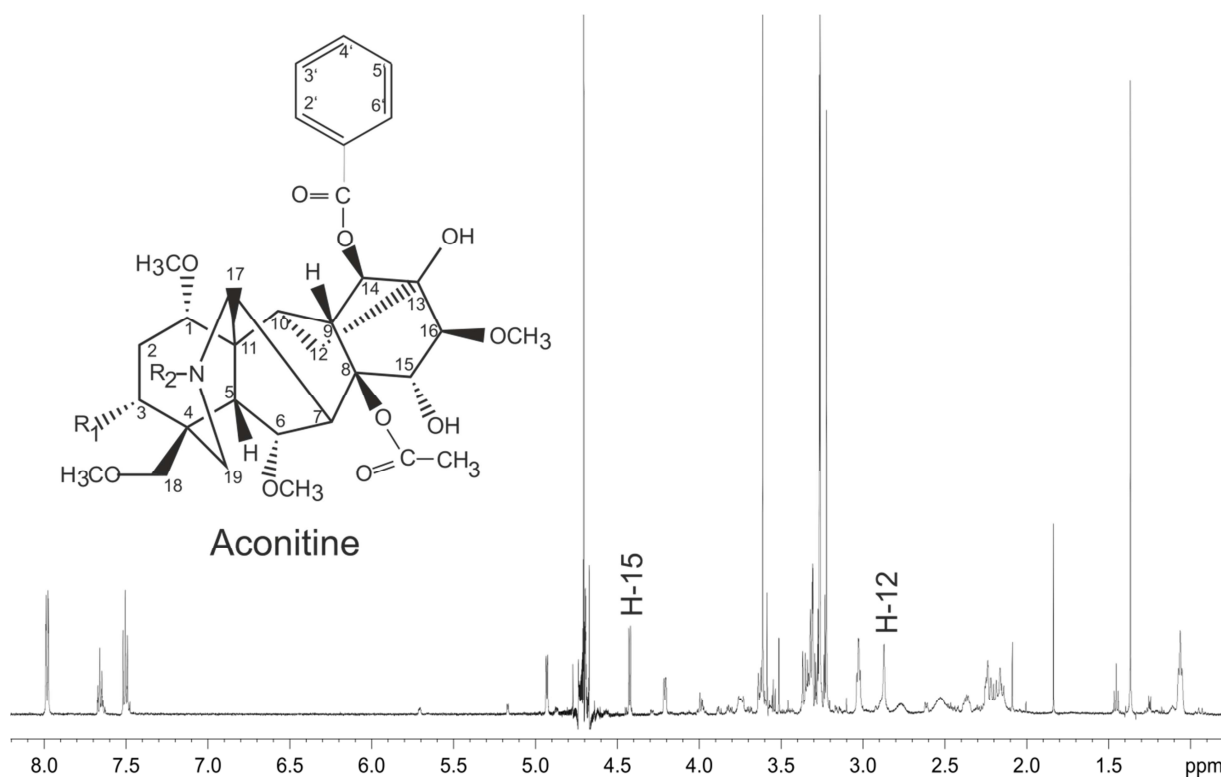


Figure 22. Chemical structure and ¹H-NMR spectra of aconitine in D₂O/MeOD (2:1). The protons H-15 and H-12 demonstrated changes of the chemical shift when the complexation was observed.

Table 19. ^1H -NMR spectra of aconitine in $\text{D}_2\text{O}/\text{MeOD}$ (2:1)

Position	Multiplicity	δ (ppm)
C-2'	CH	7.99
C-6'	CH	7.99
C-4'	CH	7.67
C-3'	CH	7.67
C-5'	CH	7.51
C-14	CH	4.93
C-15	CH	4.43
C-6	CH	4.21
C-3	CH	3.82
C-18- OCH_3	CH_3	3.61
C-18	CH_2	3.35
C-16	CH	3.29
C-1- OCH_3	CH_3	3.27
C-6- OCH_3	CH_3	3.27
C-1	CH	3.25
C-17	CH	3.24
C-16- OCH_3	CH_3	3.23
C-19	CH_2	3.13; 2.37
C-9	CH	3.05
C-12	CH_2	2.92; 2.34
C-7	CH	2.87
C-20	CH_2	2.52
C-8- OCOCH_3	CH_3	1.37
C-21	CH_3	1.06

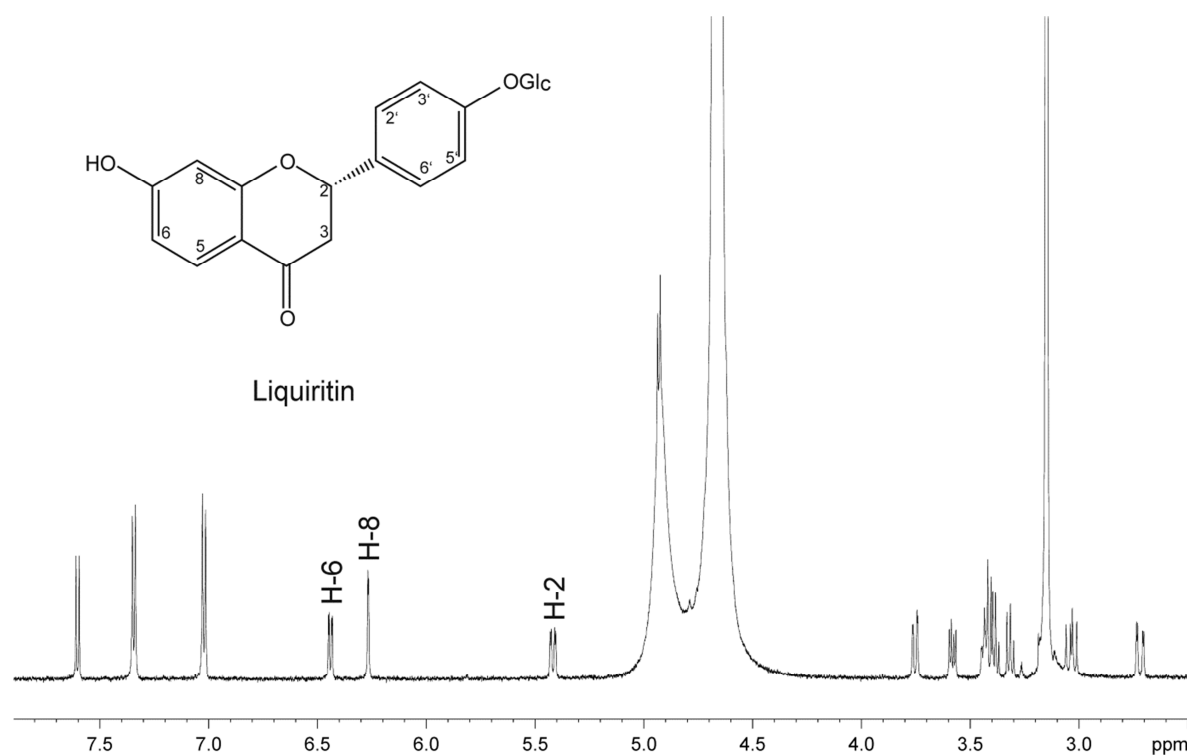


Figure 23. Chemical structure and ^1H -NMR spectra of liquiritin in $\text{D}_2\text{O}/\text{MeOD}$ (2:1). The protons H-6, H-8 and H-2 of liquiritin demonstrated changes of the chemical shift when the complexation was observed.

Table 20. ^1H -NMR spectra of liquiritin in $\text{D}_2\text{O}/\text{MeOD}$ (2:1)

Position	Multiplicity	δ (ppm)
C-5	CH	7.6
C-2'	CH	7.34
C-6'	CH	7.34
C-3'	CH	7.03
C-5'	CH	7.03
C-6	CH	6.44
C-8	CH	6.27
C-2	CH	5.42
C-6''	CH_2	3.76; 3.59
C-2''	CH	3.41
C-3	CH_2	3.04; 2.73

5.1.6.1 Determination of the stoichiometry by the continuous variations method

For measuring the stoichiometry of the complex with NMR method, Job's plot method²⁴⁸⁻²⁵⁰ was applied. Chemical shift changes of H-6 of liquiritin (6.44 ppm) were measured as a function of aconitine molar fraction with a constant sum of [aconitine] + [liquiritin] 0.5 mM (see table 21). The position of maximum at $R = 0.5$ indicates the stoichiometry of the complex 1:1 (see figure 24).

Table 21. Chemical shift changes of H-6 of liquiritin were observed and multiplied with the mole fraction of liquiritin

Shift change (Hz)	Conc. of liquiritin (mM)	Mole fraction of liquiritin	$\Delta\delta \times \text{mole fraction}$
0.0	0.00	0.00	0.0
54.1	0.13	0.27	14.6
49.0	0.20	0.40	19.6
41.4	0.25	0.50	20.7
27.5	0.30	0.60	16.5
9.3	0.37	0.73	6.8
1.8	0.43	0.87	1.6
0.0	0.50	1.00	0.0

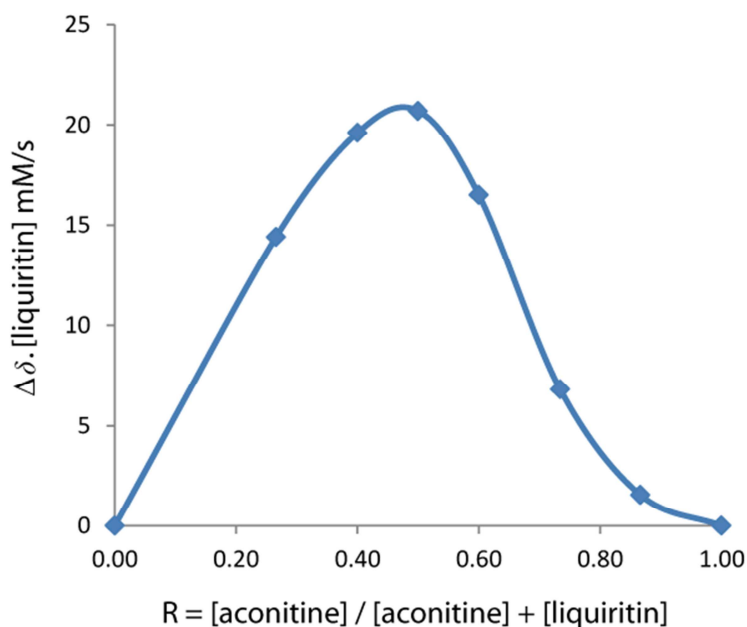


Figure 24. Job's plot of the chemical shift changes of H-6 of liquiritin ($\delta = 6.44 \text{ ppm}$) for the aconitine/liquiritin complex in $\text{D}_2\text{O}/\text{MeOD}$ (2:1). [aconitine] + [liquiritin] = 0.5 mM

5.1.6.2 Estimation of the binding constants by the ^1H -NMR titration method

The stability of the complex of aconitine and liquiritin was analyzed by monitoring the changes of selected protons of respectively, aconitine and liquiritin at a constant concentration by ^1H -NMR titrations²⁵¹. The reaction equilibrium of the complex between host (H) and guest (G) molecules is described as:



The association constant of this complex is represented by the following equation:

$$K_{nm} = \frac{[o\text{HG}]}{[\text{G}]^n[\text{H}]^m} \quad (2)$$

Two ^1H -NMR titrations were performed, in the first titration liquiritin served as host and aconitine as guest, whereas in the second they acted reversely. Ten sample solutions were prepared as the concentration of each aconitine increased from 0.0 to 4.0 mM (1:0 to 1:20) versus a constant concentration of liquiritin (0.2 mM). $\Delta\delta_{\text{obs}}$ of H-2 (5.42 ppm), H-6 (6.44 ppm) and H-8 proton (6.23 ppm) of liquiritin (figure 25) was plotted against the concentration of aconitine, where $\Delta\delta_{\text{obs}}$ is the difference between the observed ^1H -NMR chemical shift of liquiritin at each titration point and the ^1H -NMR chemical shift of free liquiritin. In figure 26 a change in binding behavior occurred as the concentration of aconitine increased. At a higher concentration of aconitine the influence of self-association might need to be taken into account which is the result of its solubility limit, which starts at 1.2 mM in $\text{D}_2\text{O}/\text{MeOD}$ (2:1) mixture. Therefore, only the titration points from 0.0 mM to 1.2 mM were used for the analysis of the binding constants (figure 27).

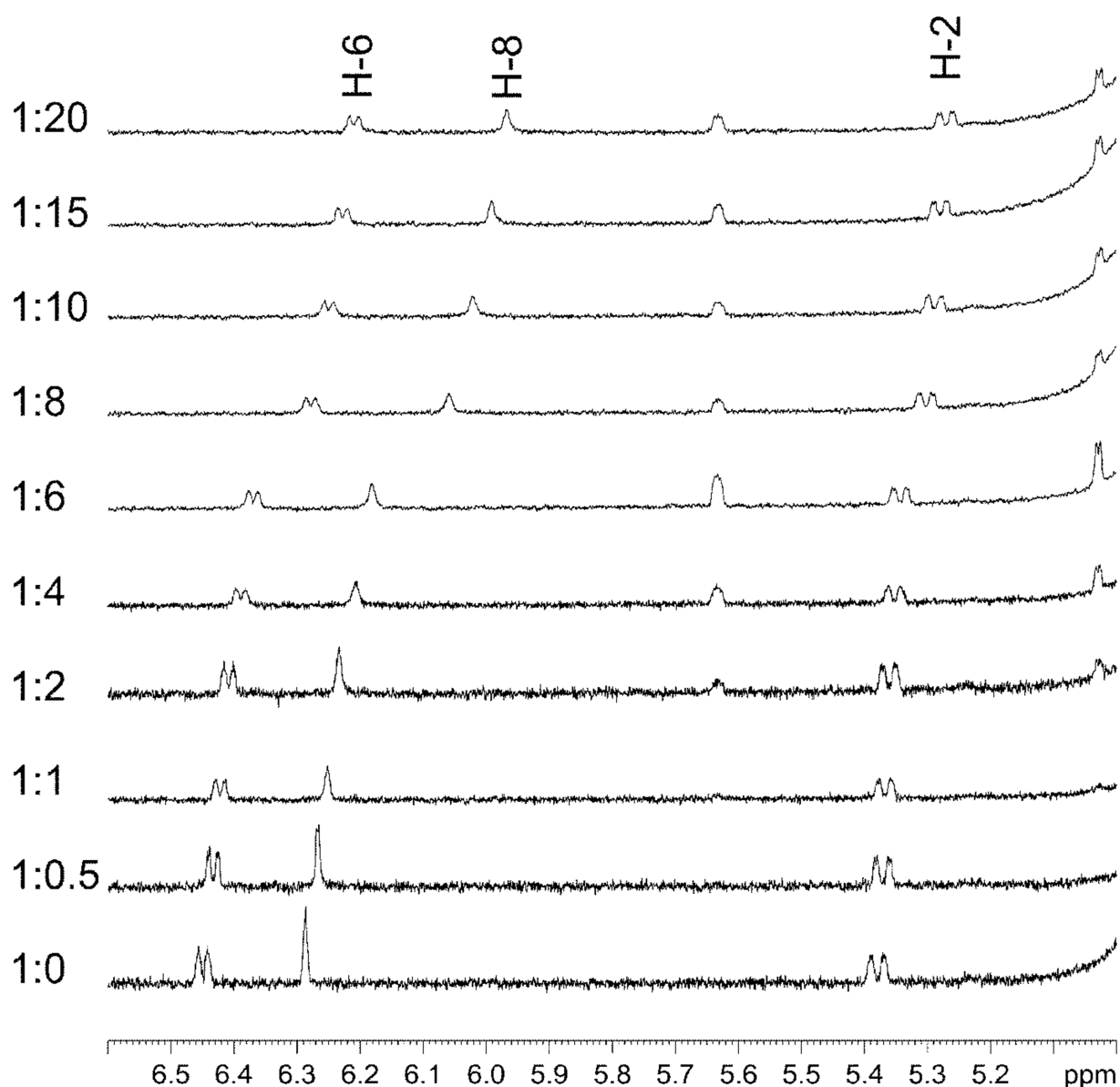


Figure 25. Section of ^1H -NMR titration spectra demonstrating the chemical shifts of H-6, H-8 and H-2 of free liquiritin (0.2 mM) (bottom of the figure) and the observation of the shift changes of these protons at each titration point (0.0, 0.1, 0.2, 0.4, 0.8, 1.2, 1.6, 2.0, 3.0, 4.0 mM aconitine, on the left side of the figure see ratio of liquiritin:aconitine) in $\text{D}_2\text{O}/\text{MeOD}$ (2:1).

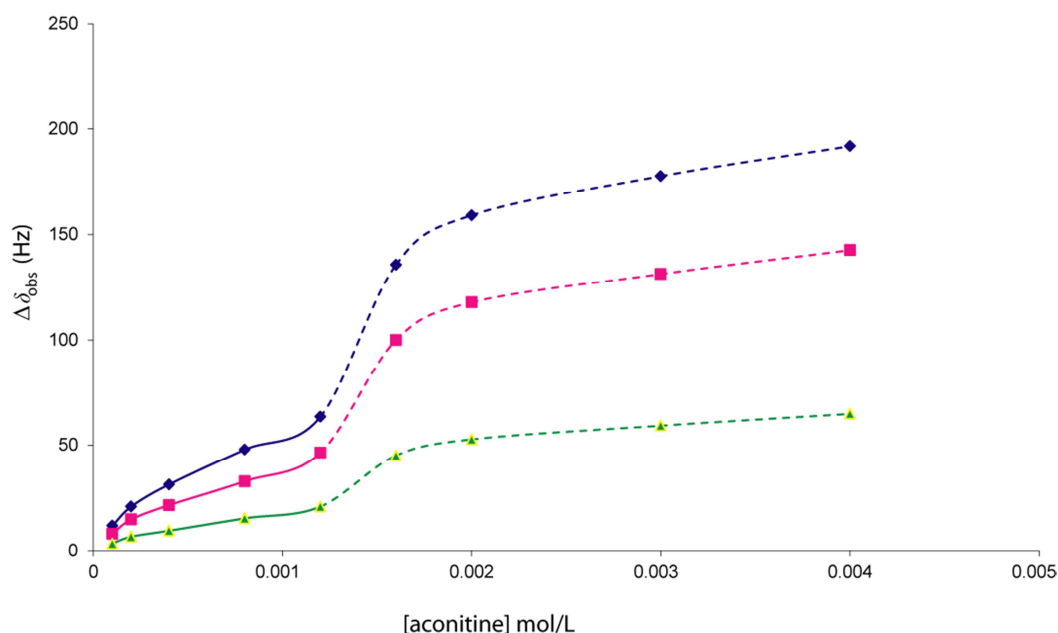


Figure 26. Titration curve of aconitine/liquiritin. $\Delta\delta_{\text{obs}}$ (Hz) = difference between ^1H -NMR shift of H-8 (■), H-6 (▲) and H-2 (◆) of liquiritin (constant concentration of 0.2 mM) detected at each titration point and that of free liquiritin without aconitine. The broken line demonstrates the self-aggregation of aconitine starting at a concentration between 0.8 mM and 1.2 mM. The experiment was carried out in a $\text{D}_2\text{O}/\text{MeOD}$ (2:1) mixture with a liquiritin concentration of 0.2 mM.

An addition of aconitine between 0 and 4 mM to 0.2 mM liquiritin in $\text{D}_2\text{O}/\text{MeOD}$ (2:1) was seen to cause a shift change from 64.8 Hz (H-2), 142.6 Hz (H-6) to 191.8 Hz (H-8) (table 22).

The value of K can be obtained by curve-fitting²⁵²⁻²⁵⁴ that describes fast exchange systems^{255,256}.

$$\Delta\delta_{\text{obs}} = 0.5 \Delta\delta_{\text{max}} \frac{[(H + G + (1/K)) - [(H + G + (1/K))^2 - 4HG]^{1/2}]}{H} \quad (3)$$

This equation (3) shows the curve-fitting procedure of the experimental concentration dependence for the 1:1 complex results in accordance with the observed shift changes (figure 27). This allows us to conclude a complex formation with stoichiometry 1:1 and a calculated binding constant of $K = 3000 \pm 300$ in $\text{D}_2\text{O}/\text{MeOD}$ (2:1).

Table 22. Shift changes of H-8 (6.23 ppm), H-6 (6.44 ppm) and H-2 (5.42 ppm) of liquiritin at a concentration of 0.2 mM/L in D₂O/MeOD (2:1); $\Delta\delta_{\max} = 21$; calculated binding constant of 3000 ± 300 (L/mol)

[Aconitine] (mol/L)	$\Delta\delta$	Shift change (Hz)		
		H-8	H-6	H-2
0.0001	5.66	11.9	8.21	3.6
0.0002	9.71	20.9	14.9	6.8
0.0004	14.26	31.3	21.6	9.6
0.0008	17.54	47.8	32.8	15.4
0.0012	18.71	63.4	46.3	20.9
0.0016	19.30	135.8	100.0	45.0
0.002	19.65	159	117.9	52.7
0.003	20.11	177.6	131.4	59.2
0.004	20.33	191.8	142.6	64.8

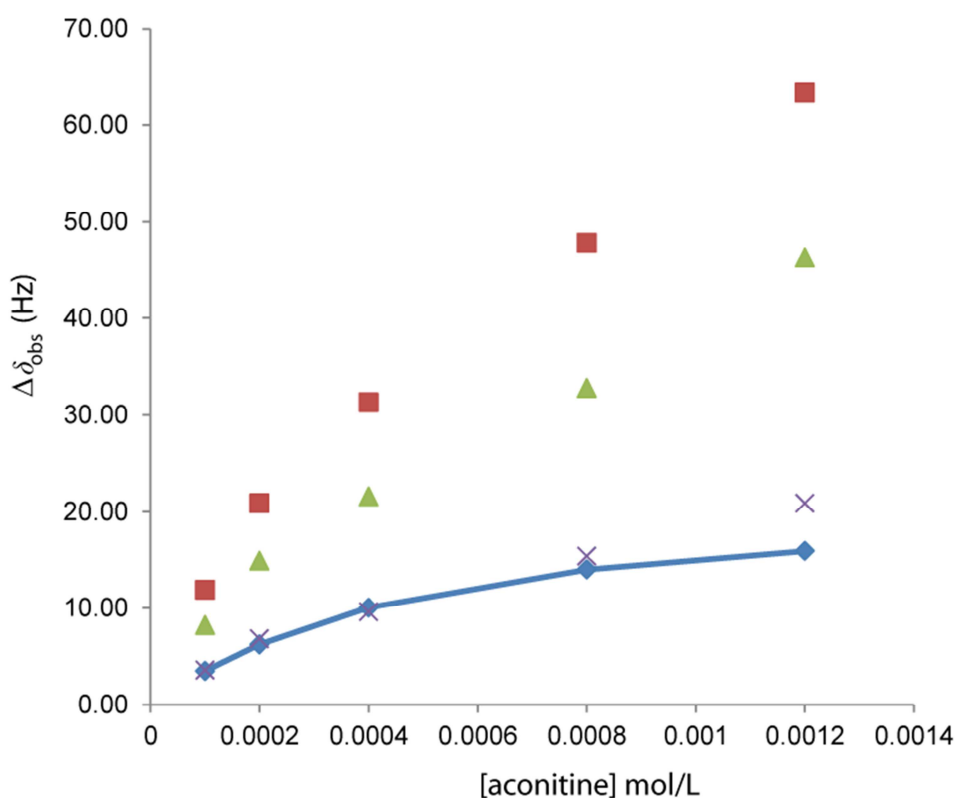


Figure 27. Section of titration curve of aconitine/liquiritin. $\Delta\delta_{\text{obs}}$ (Hz) = difference between ¹H-NMR shift of H-8 (■), H-6 (▲) and H-2 (×) of liquiritin (constant concentration of 0.2 mM in D₂O/MeOD (2:1)) detected at the first five titration points and that of free liquiritin without aconitine. $\Delta\delta_{\max}$ (◆) was obtained by curve fitting procedure using equation 3.

All titration points of H-2 fitted the equation (equation 3), giving the corresponding binding constant $K = 3000 \pm 300$ L/mol in D₂O/MeOD (2:1). The experimental point at a concentration of 1.2 mM of aconitine was not taken into account due to the appearance of self-aggregation of aconitine.

The same analysis was repeated inversely with a constant concentration of aconitine and a varying concentration of liquiritin to avoid the self-aggregation of aconitine. Chemical shift changes ($\Delta\delta_{\text{obs}}$) of H-12 (2.87 ppm) (figure 28) and H-15 (4.42 ppm) at each titration point (table 23) were observed and resulted in a maximal shift change from 8 Hz (H-12) to 20 Hz (H-15).

A stability constant of 4000 ± 600 L/mol in D₂O/MeOD (1:2) was calculated by means of equation 3 (figure 29).

Table 23. Shift changes of H-12 (2.87ppm) and H-15 (4.42 ppm) of aconitine at a concentration of 0.2 mM/L in D₂O/MeOD (1:2); $\Delta\delta_{\text{max}} = 21$; calculated binding constant of 4000 ± 600 (L/mol)

[Liquiritin] (mol/L)	$\Delta\delta$	Shift change (Hz)	
		H-15	H-12
0.0001	6.15	3.5	2
0.0002	10.50	9	3.5
0.0004	15.10	11	5
0.0008	18.11	14	6
0.0012	19.12	16	7
0.0016	19.61	18	8
0.002	19.90	19	8
0.003	20.28	20	8
0.004	20.46	20	8

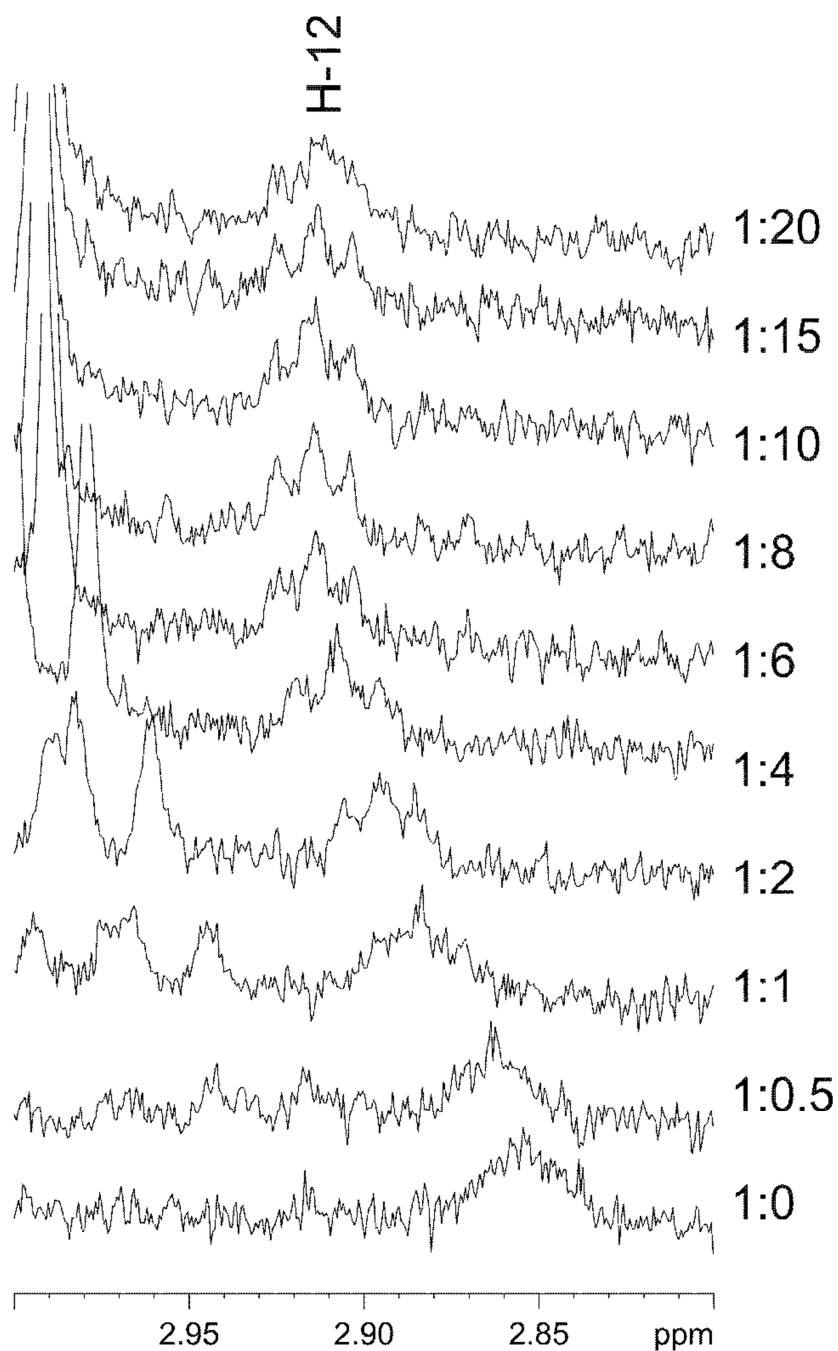


Figure 28. Section of ^1H -NMR titration spectra demonstrating the chemical shift of H-12 of free aconitine (0.2 mM) (bottom of the figure) and the observation of the shift changes of this proton at each titration point (0.1, 0.2, 0.4, 0.8, 1.2, 1.6, 2.0, 3.0, 4.0 mM liquiritin, on the right side of the figure see ratio of aconitine:liquiritin) in $\text{D}_2\text{O}/\text{MeOD}$ (1:2).

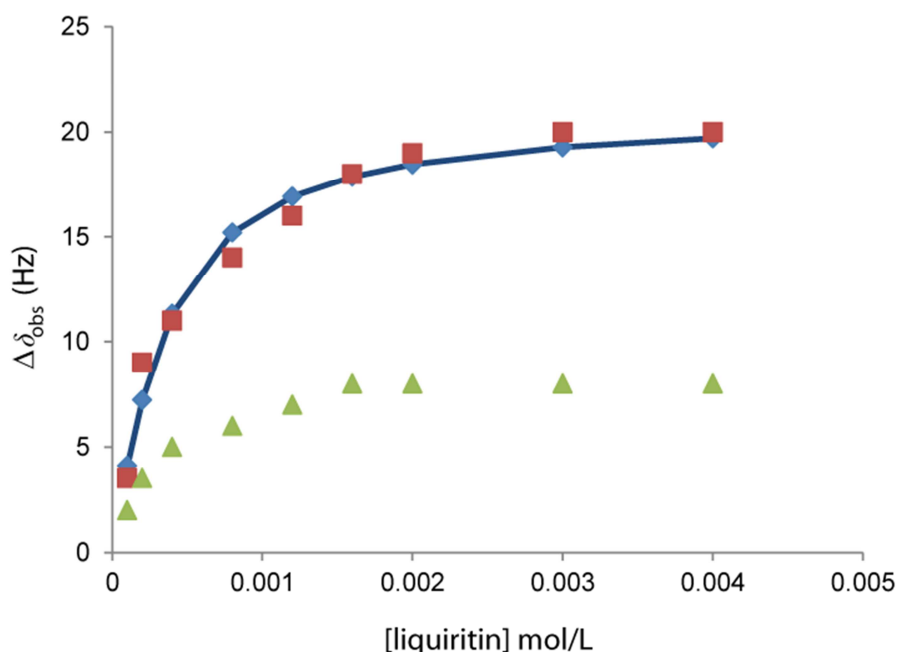


Figure 29. Titration curve of liquiritin/aconitine. $\Delta\delta_{\text{obs}}$ (Hz) = difference between ^1H -NMR shift of H-15 (■) and H-12 (▲) of aconitine (constant concentration of 0.2 mM in $\text{D}_2\text{O}/\text{MeOD}$ (1:2)) detected at each titration point and that of free liquiritin without aconitine. $\Delta\delta_{\text{max}}$ (◆) was obtained by curve fitting procedure using equation 3.

5.1.6.3 Structure identification of the formed complex by 2D-ROESY experiment

The 2D ROESY experiment did not show distinct dipolar couplings between the two molecules, which form the complex (figure 30). This behavior was probably caused by a relative short binding time, not sufficient for the intermolecular magnetization transfer. Consequently the dissociation process appears quite fast with k_{off} rates, which were distinctly higher than 50 s^{-1} . Such a k_{off} value was comparable to dissociations constant in a micro- to milli-molar range and therefore fitted in well to the pattern of binding-constants determined. Nevertheless, some of the liquiritin protons showed reasonably large shift changes within the job's plot measurements and during the determination of binding constants also showed small intermolecular NOEs (figure 31). H-2 of liquiritin showed weak anti-phase crosspeaks with H-20ab and H-18ab of aconitine, while H-8 of liquiritin had no detectable NOE. H-6 of liquiritin also showed a very

weak dipolar coupling to the 1-OMe group of aconitine. However, these few NOEs were not sufficient to determine structural details of the binding.

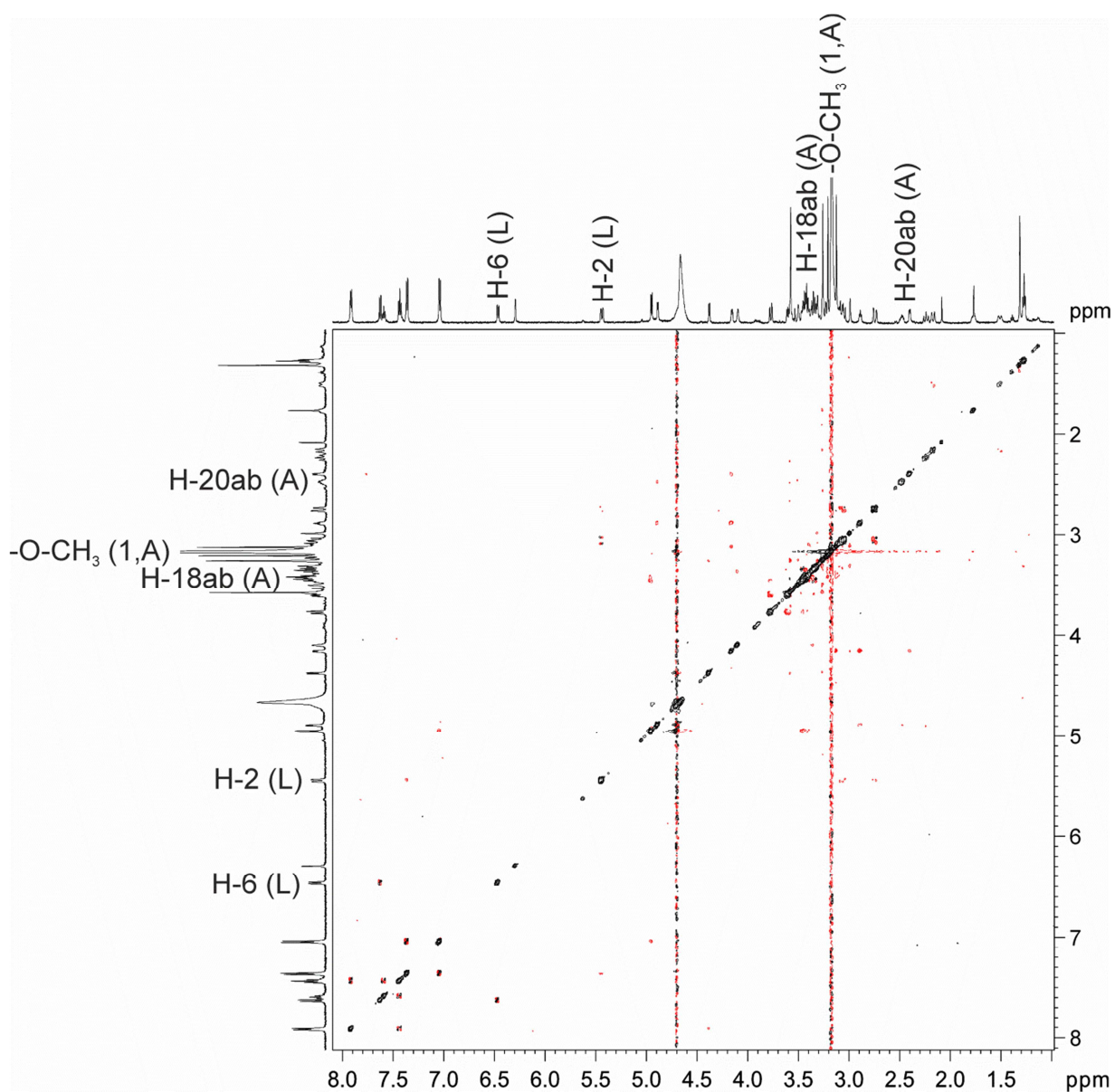


Figure 30. 2D ROESY spectra of a 1:1 mixture of aconitine (0.25 mM) and liquiritin (0.25 mM) in D₂O/MeOD (2:1)

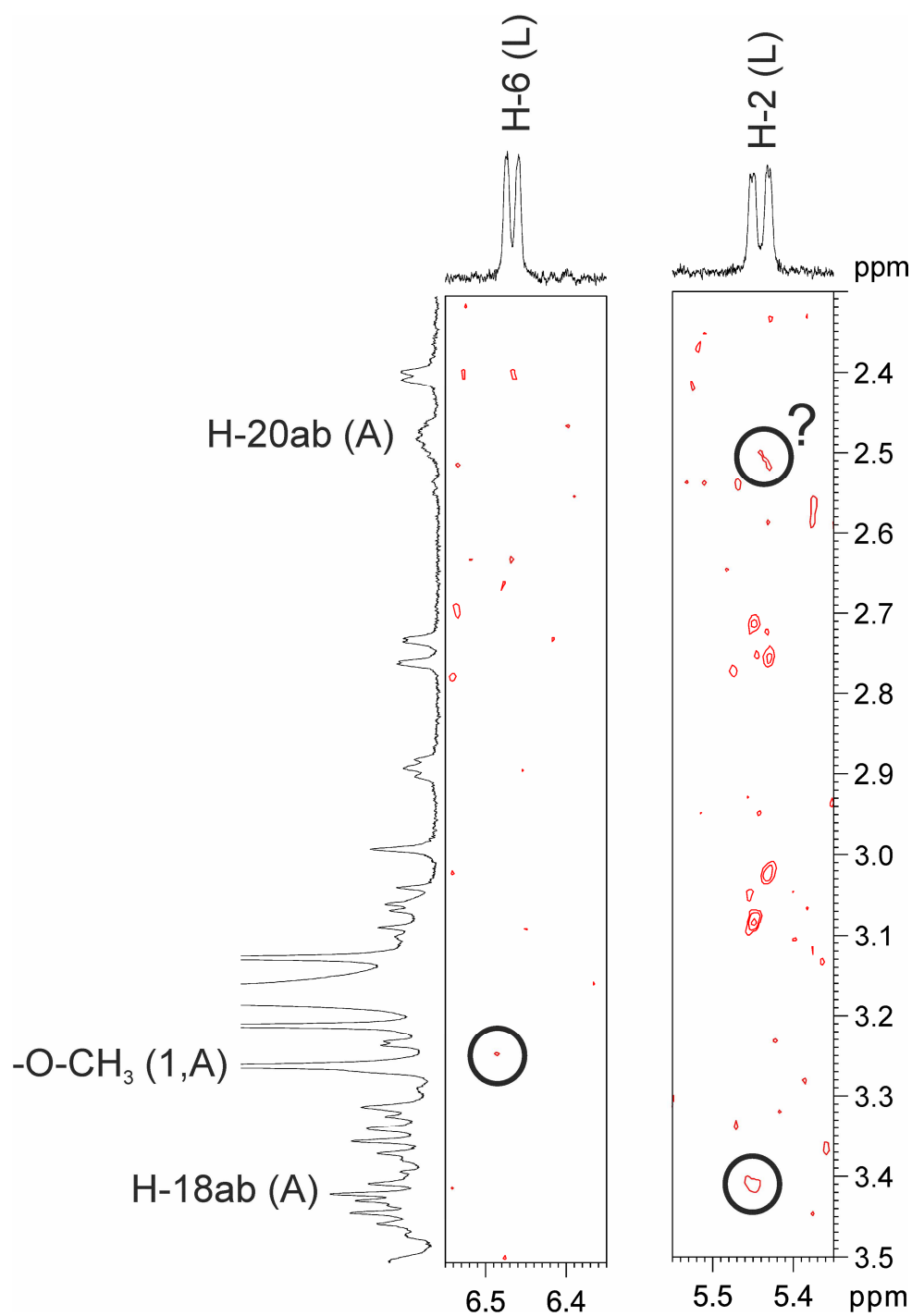


Figure 31. Section of 2D ROESY spectra of a 1:1 mixture of aconitine (0.25 mM) and liquiritin (0.25 mM) in $D_2O/MeOD$ (2:1); pointed out in the circles: weak anti-phase crosspeaks of H-2 of liquiritin (L) with H-18ab and H-20ab of aconitine (A) and weak dipolar coupling of H-6 of liquiritin (L) to 1-OMe of aconitine (A).

5.1.6.4 Determination of binding constants by UV-VIS measurements

UV-VIS absorption measurement was applied to analyze the same samples used for the estimation of the binding constants by NMR. The focus of this analysis was on examining the structural change and on obtaining further information on the complex formation by observing the peak maxima. Different optical absorption spectra of liquiritin and aconitine are shown respectively in figures 32 and 34. The spectra of liquiritin showed maxima at 214, 227, 272 and 308 nm and therefore an overlap occurred with some maxima of aconitine (232 and 274 nm). Because of this, only the maxima at 214 nm and 308 nm of liquiritin were suitable for studying the shift changes. There were no changes observed in the shape of the UV spectra, either of liquiritin or of aconitine. All the spectra represented in figure 32 are combined in one graph and illustrated in figure 33 to see possible peak shifts. The monitored peak at 308 nm shifted slightly in the presence of aconitine, but was not calculable because of small device resolution. The UV-spectra 9 of figure 33 represented an outlier - there was an additional structure in the solution, which showed an absorption-maximum at 216 nm. The maxima of liquiritin at 214 nm shifted to 218 nm with an increase of the concentration of aconitine to a fourfold of liquiritin (figure 33, 1-5) whereas from a six fold concentration of aconitine added to liquiritin, this maxima disappeared (figure 33, 6-10).

5 Results

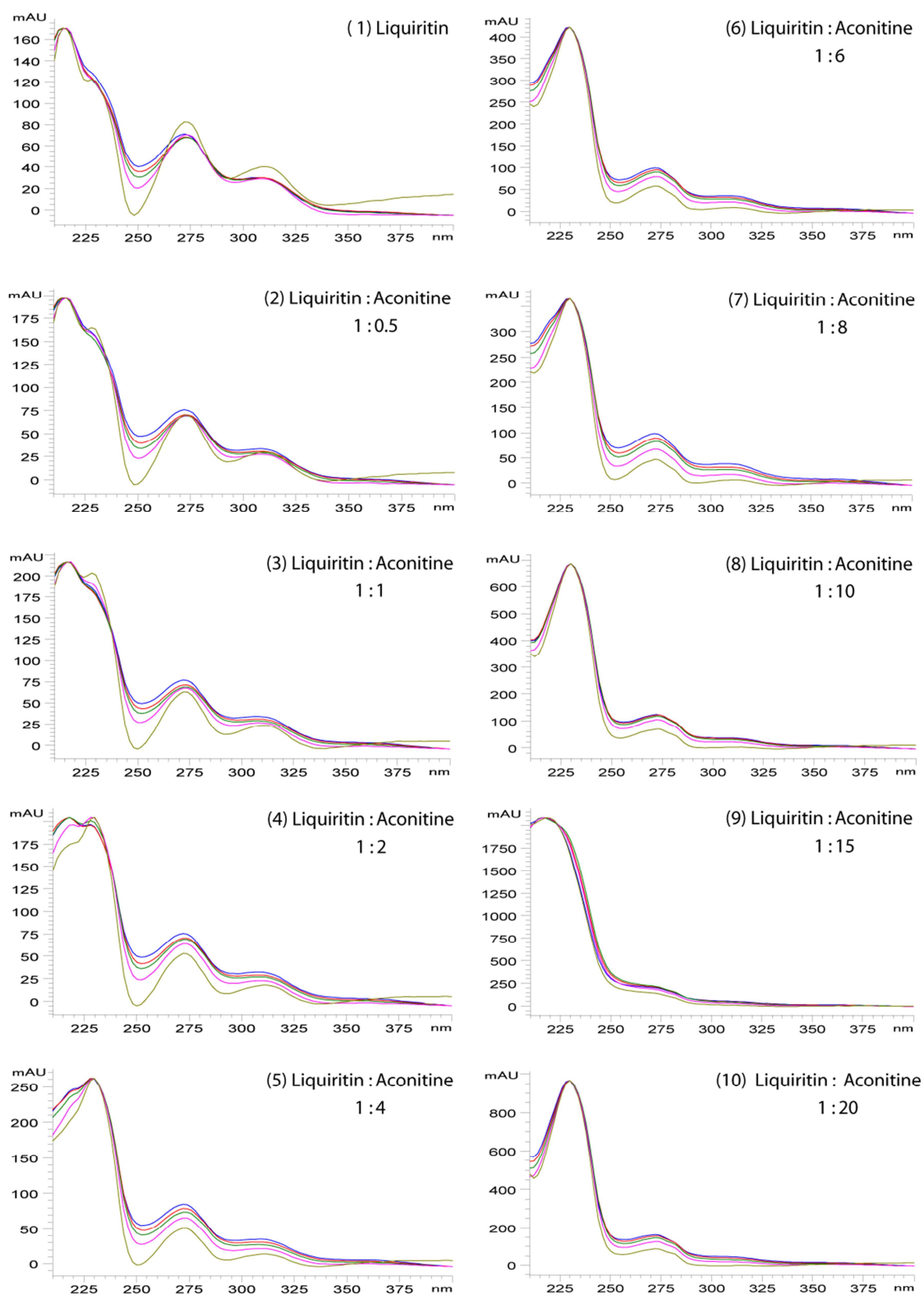


Figure 32. UV spectra of a constant concentration of liquiritin in absence and presence of different concentrations of aconitine; monitoring wavelength 233 nm.

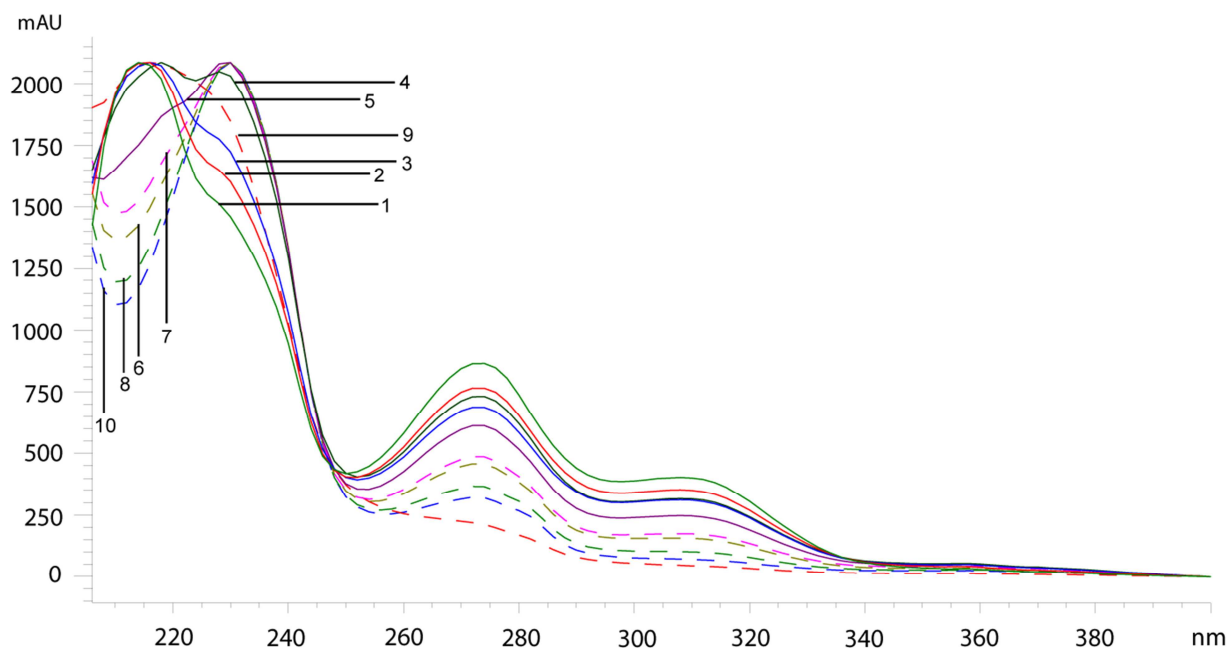


Figure 33. UV spectra shown in figure 32 combined in one graph; constant concentration of liquiritin in absence and presence of different concentrations of aconitine; (1) 1:0, (2) 1:0.5, (3) 1:1, (4) 1:2, (5) 1:4, (6) 1:6, (7) 1:8, (8) 1:10, (9) 1:15, (10) 1:20; observed maxima at 308 nm.

Figure 34 shows the repetition of the experiment under different conditions. By observing the maxima of aconitine at a constant concentration while increasingly high concentrations of liquiritin were added, no shift change could be determined. As already mentioned above, there was an overlay of the maxima of aconitine (232 and 274 nm) by two maxima of liquiritin (227 and 272 nm) and thus a peak shift could not be detected.

5 Results

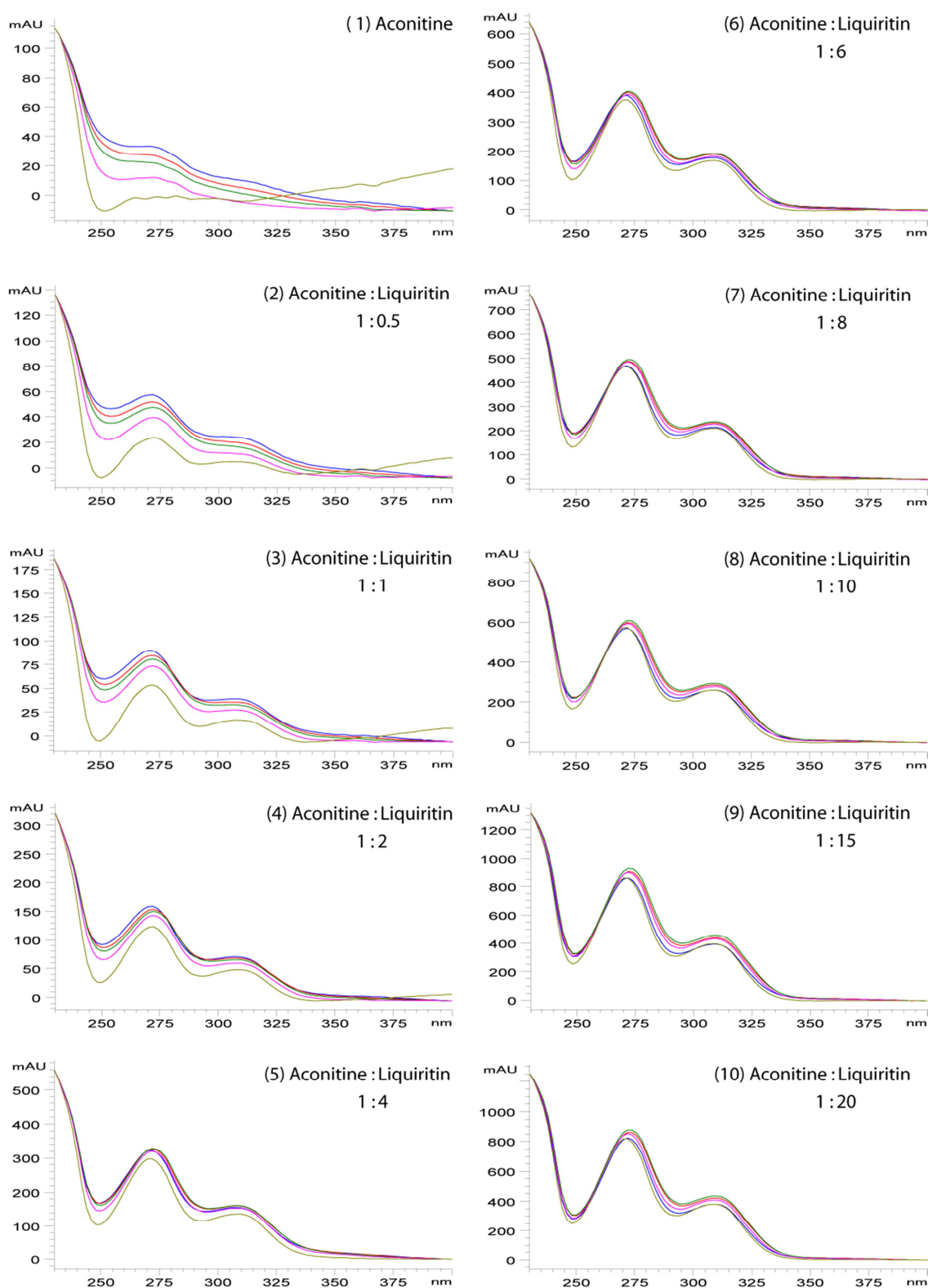


Figure 34. UV spectra of a constant concentration of aconitine in absence and presence of different concentrations of liquiritin; monitoring wavelength 233 nm.

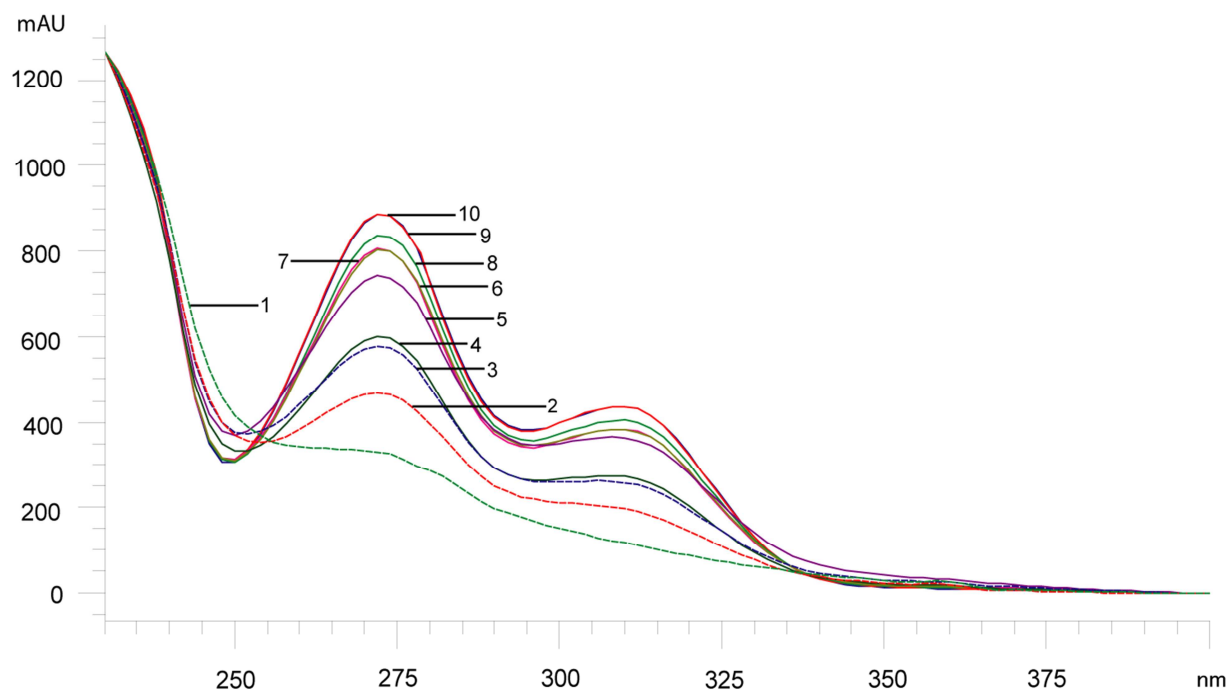


Figure 35. UV spectra shown in figure 34 combined in one graph; constant concentration of aconitine in absence and presence of different concentrations of liquiritin; (1) 1:0, (2) 1:0.5, (3) 1:1, (4) 1:2, (5) 1:4, (6) 1:6, (7) 1:8, (8) 1:10, (9) 1:15, (10) 1:20

5.1.6 Conclusion of the phytochemical analysis

The concentrations of arsenic, lead, cadmium and mercury determined in the herbal material of SNT are lower than the permissible levels. MeOH was found to be the most effective extraction solvent and the HPLC-DAD method of analysis showed good repeatability, precision and recovery. Small amounts of HA (88.80 $\mu\text{g/g}$, 72.47 $\mu\text{g/g}$) were detected in two of eight processed batches of *A. carmichaelii* extracted with MeOH. HA concentrations up to 269.34 $\mu\text{g HA/g}$ were detected in the unprocessed batch of *A. carmichaelii*. None of the aconitines could be detected in the decoction of processed *A. carmichaelii* and SNT decoction containing unprocessed *A. carmichaelii*. HA was detected in the decoction of unprocessed *A. carmichaelii* less than one fourth of the amount detected in the extraction with MeOH. The studies on herbal interactions led to the conclusion that unprocessed *A. carmichaelii* combined with *G. uralensis* showed the biggest alteration in the concentration of HA, which was reduced to an undetectable level. Next followed *C. cassia* and then *Z. officinale*, which both showed a significant reduction of HA per

mL decoction. It was also observed that liquiritin and isoliquiritin interact with AC, HA and MA and that AC, HA and MA contents were reduced to an undetectable level using a 10-15 fold concentration of isoliquiritin. Another interesting observation was made: in the last part of the peak of isoliquiritin in the mixture with VDR1, an altered UV-spectra, resembling a mixture of the UV-spectra of the aconitines and that of isoliquiritin, could be seen.

Due to a relatively short binding time of the complex formed, it was not possible to determine structural details of the binding between aconitine and liquiritin. Nevertheless, the analysis using the job plot method and curve fitting allowed conclusions to be drawn on the complex formation with stoichiometry 1:1 and a calculation of the stability of the complex, $K = 3000 \pm 300$ L/mol for liquiritin/acnitrine in D₂O/MeOD (2:1) and $K = 4000 \pm 600$ L/mol in D₂O/MeOD (1:2) for acnitrine/liquiritin.

Precise information about structural changes and complex formation could not be obtained using UV-VIS. The peak maxima of liquiritin monitored at 308 nm shifted slightly in the presence of acnitrine, but could not be calculated because of less device resolution.

5.2 In vivo experiments

In the recent study, acute myocardial infarction (AMI) was induced in both male and female rats by left anterior descending artery ligation. The forty-eight infarcted rats were randomly divided into four groups: SNT-high dosage group (SNT-HD), SNT-low dosage group (SNT-LD), Fosinopril group (FPS) and the model group (model) (table 24). Twenty-four rats served as a control group, a sham-operation group (SH-O) underwent a sham operation, consisting of thoracotomy and cardiac exposure but without coronary artery ligation, and the blank group (normal group) didn't receive any operation or treatment.

Table 24. The six experimental groups for examining the effects of SNT on early ventricular remodeling in rats post-myocardial infarction in vivo

Group	N*	Operation	Oral administration	N**
Normal	12	-	-	12
SH-O	12	Sham-operation	Drinking water	12
Model	12	AMI	Drinking water	12
FPS	12	AMI	Fosinopril (900 µg/kg)	12
SNT-LD	12	AMI	Low dose SNT (4.5 g/kg)	12
SNT-HD	12	AMI	High dose SNT (13.5 g/kg)	10

N* = number of animals starting the experiment, in each case six male and six female rats

N** = number of animals after 30 days post-MI, in each case half male and half female rats

One female and one male rat of the SNT-HD-group died in the third, respectively the fourth week during the experiment. Two female rats of the FPS-group and one female of the model group died during anesthetization prior to echocardiography on the last day. Echocardiographic determination was still conducted on these three rats and tissue samples were also collected. For the two missing body weight values of the SNT-HD group after weeks three and four, the mean body weight of the remaining ten rats in this group was used.

5.2.1 Effect of treatment with SNT on body weight and heart weight

In the current study, the body weight of each group was calculated and compared between the different groups (table 25). The rats weighed between 180–200 g at their delivery. The graph (figure 36) represents the female and male rats of each group throughout the experimental period. There was no statistically significant difference between the mean body weights of each single group. However, a great difference could be observed among female and male rats: both gained weight during the experiment, whereas the male rats showed a notably higher increase in weight compared to the female rats. The rats of the normal group were only weighed prior to death.

Table 25. Effect of the treatment with SNT for 30 days on the body weight in myocardial-infarct rats

Animal group	after 5 days	after 10 days	after 15 days	after 20 days	after 25 days	day of death
Model						
Mean female	189.00	214.17	235.00	259.33	271.67	246.67
Mean male	210.33	243.33	280.83	327.00	364.00	341.33
RSD female	8.39	6.72	11.90	6.07	9.48	7.45
RSD male	4.82	12.13	10.17	12.95	17.44	13.06
FPS						
Mean female	184.67	205.83	225.83	244.67	262.33	239.00
Mean male	192.67	250.00	295.00	326.00	365.33	324.33
RSD female	7.09	9.75	7.86	12.42	9.96	13.13
RSD male	9.34	13.84	20.62	27.71	32.01	48.96
SNT-LD						
Mean female	187.17	208.33	221.67	239.33	254.67	239.67
Mean male	191.67	212.50	269.17	299.33	363.33	345.00
RSD female	6.79	11.06	14.91	15.30	17.80	17.68
RSD male	14.62	17.26	27.45	29.66	43.98	46.51
SNT-HD						
Mean female	190.00	201.67	224.17	282.00	264.40	256.80
Mean male	194.17	223.50	269.80	310.40	332.00	316.00
RSD female	9.57	6.24	6.72	52.70	9.16	19.37
RSD male	13.72	21.09	9.17	14.55	21.54	17.10
SH-O						
Mean female	179.17	187.67	210.83	230.33	244.67	223.33
Mean male	201.33	218.33	260.00	312.33	349.67	344.33
RSD female	7.86	7.67	7.86	8.12	9.91	7.74
RSD male	10.80	9.86	34.03	36.19	33.83	31.02

n = 12; RSD is the relative standard deviation

Model = model group (induction of AMI, treatment: water)

FPS = fosinopril group (induction of AMI, treatment: 900 µg/kg fosinopril)

SNT-LD = SNT-low dosage group (induction of AMI, treatment: 4.5 g/kg SNT)

SNT-HD = SNT-high dosage group (induction of AMI, treatment: 13.5 g/kg SNT)

SH-O = sham-operated group (sham-operation, treatment: water)

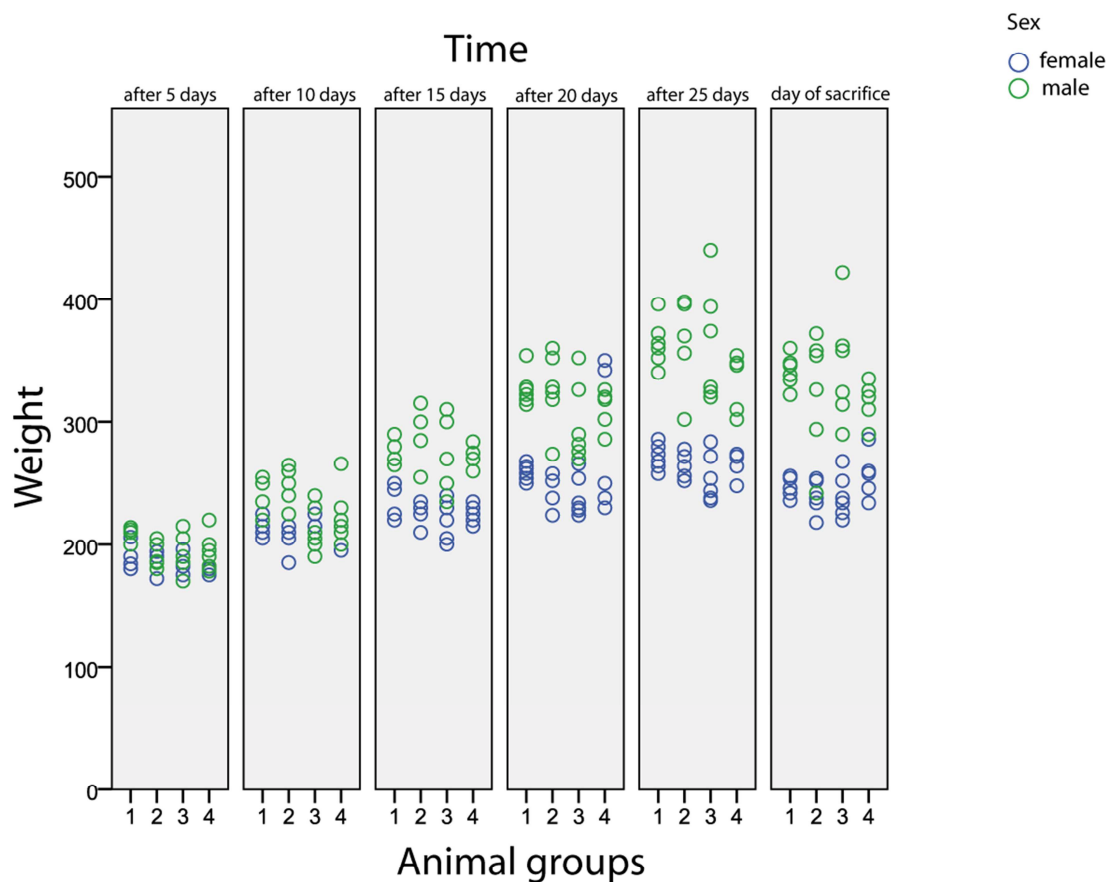


Figure 36. Effect of the treatment with SNT for 30 days on the body weight in myocardial-infarct rats

No significant difference was found among the individual groups

n = 12

1 = Model group (induction of AMI, treatment: water)

2 = Fosinopril group (induction of AMI, treatment: 900 μ g/kg fosinopril)

3 = SNT-low dosage group (induction of AMI, treatment: 4.5 g/kg SNT)

4 = SNT-HD = SNT-high dosage group (induction of AMI, treatment: 13.5 g/kg SNT)

5.2.1.1 Body weight/wet heart weight ratio

The body weight/wet heart weight ratio was calculated according to Kim et al.²⁶⁹. At this point, ten rats of each group were randomly selected for further analysis. In the SNT-HD group only ten rats survived the experimental period. This has to be taken into consideration, as it could possibly influence the test results due to the fact that all rats of the SNT-HD group have been included in the experiment, whereas in the other groups the rats were randomly selected.

There was no statistical difference observed in the heart/body weight ratio between the individual groups (see figure 37 and table 26). As could be expected, the normal and SH-O group showed a remarkably lower ratio and the statistical spread was smaller than in the other groups. The female rats had in total notably higher heart/body weight ratios due to less body weight, but heart weights comparable to those of the male rats.

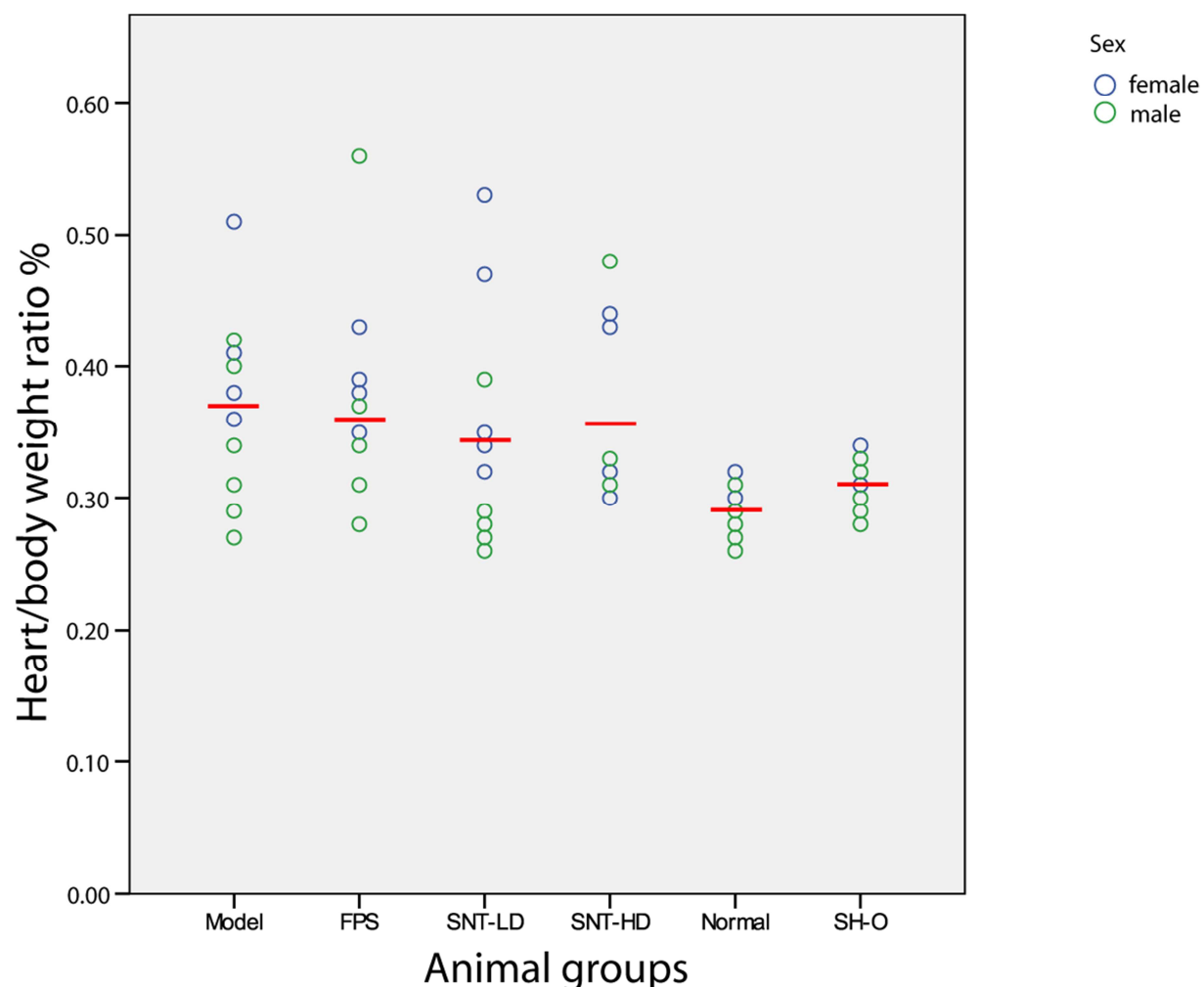


Figure 37. Effect of 30 days' treatment with SNT on the heart/body weight ratio

No significant difference was found among the individual groups.

n = 10

Model = model group (induction of AMI, treatment: water)

FPS = fosinopril group (induction of AMI, treatment: 900 µg/kg fosinopril)

SNT-LD = SNT-low dosage group (induction of AMI, treatment: 4.5 g/kg SNT)

SNT-HD = SNT-high dosage group (induction of AMI, treatment: 13.5 g/kg SNT)

Normal = blank group (no operation, no treatment)

SH-O = sham-operated group (sham-operation, treatment: water)

Table 26. Effect of the treatment with SNT for 30 days on the heart/body weight ratio

Animal groups	N	Minimum	Maximum	Mean	Std. Deviation	Variance
Model	12	0.27	0.51	0.37	0.07	0.00
FPS	12	0.28	0.56	0.36	0.08	0.01
SNT-LD	12	0.26	0.53	0.35	0.08	0.01
SNT-HD	10	0.30	0.48	0.36	0.07	0.00
Normal	12	0.26	0.32	0.29	0.02	0.00
SH-O	12	0.28	0.34	0.31	0.02	0.00

Model = model group (induction of AMI, treatment: water)

FPS = fosinopril group (induction of AMI, treatment: 900 µg/kg fosinopril)

SNT-LD = SNT-low dosage group (induction of AMI, treatment: 4.5 g/kg SNT)

SNT-HD = SNT-high dosage group (induction of AMI, treatment: 13.5 g/kg SNT)

Normal = blank group (no operation, no treatment)

SH-O = sham-operated group (sham-operation, treatment: water)

5.2.2 Echocardiographic analysis

Echocardiography was performed post-MI on the 31st day (see figure 38). The values were calculated on an average of five cycles. The rats of the model group showed increased left ventricle end-diastolic dimensions (LVDd) and left ventricle end-systolic dimensions and reduced left ventricular ejection fraction (EF) (see table 27 and figure 39).

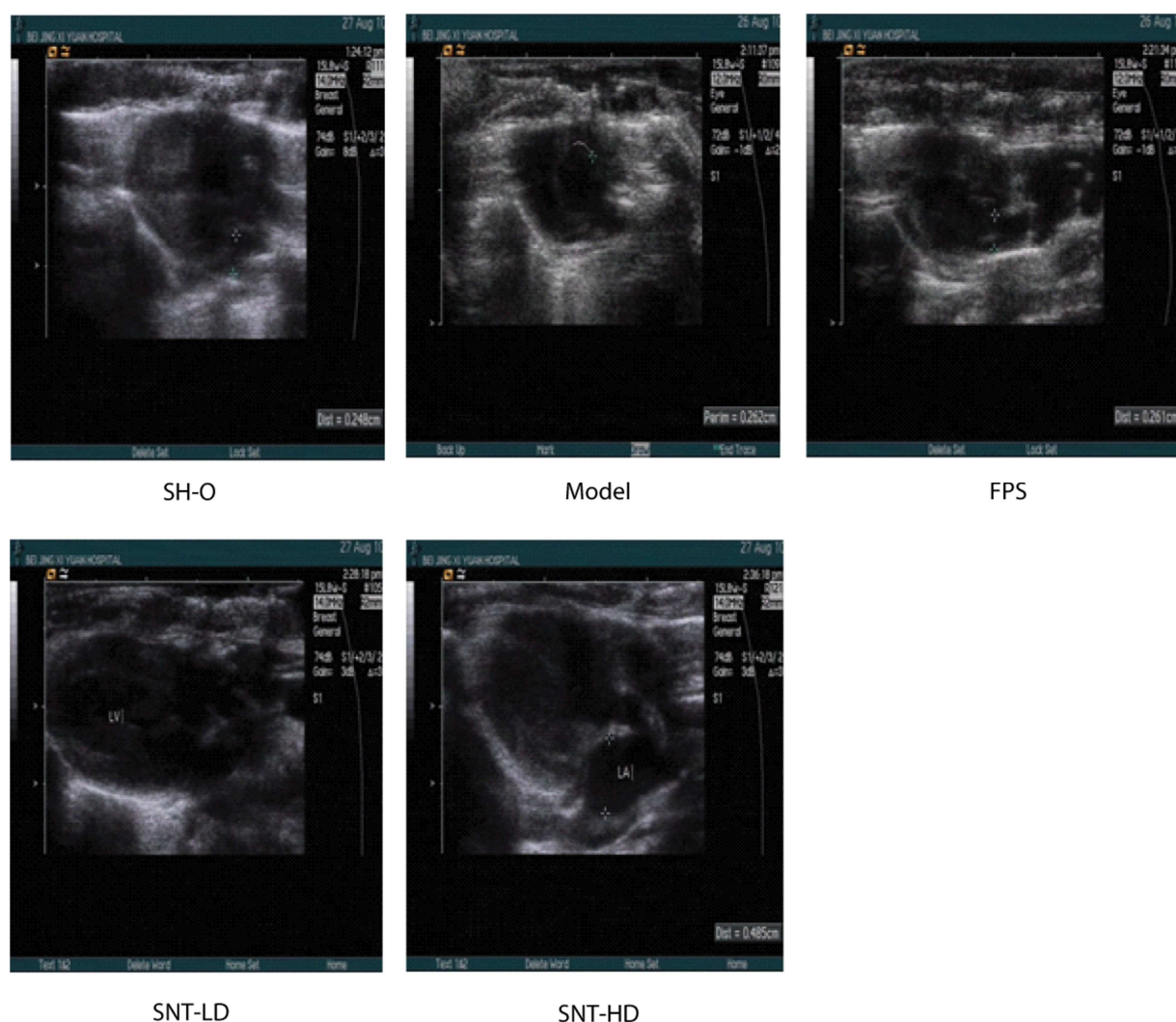


Figure 38. Effects of the treatment with SNT on the echocardiographic findings

SH-O = sham-operated group (sham-operation, treatment: water)

Model = model group (induction of AMI, treatment: water)

FPS = fosinopril group (induction of AMI, treatment: 900 μ g/kg fosinopril)

SNT-LD = SNT-low dosage group (induction of AMI, treatment: 4.5 g/kg SNT)

SNT-HD = SNT-high dosage group (induction of AMI, treatment: 13.5 g/kg SNT)

On intergroup analysis of the LVDD, we observed an increase in LVDD in the Model, FPS, SNT-LD and SNT-HD groups compared with the SH-O group, but only the FPS group showed a significant difference ($P < 0.05$) to the SH-O group (table 27, figure 39). A significant difference in the increase of LVDs was observed when comparing the FPS, SNT-LD and SNT-HD vs. SH-O groups (3.81 ± 1.21 , 4.95 ± 1.95 and 3.74 ± 1.47 mm vs. 0.91 ± 0.20 mm, respectively). A decrease in left ventricular EF was identified in all groups compared to the SH-O group, however

statistical differences were found in the FPS, SNT-LD and SNT-HD groups (96.90 ± 22.2 % vs. 78.03 ± 10.70 , 69.69 ± 13.91 and 77.83 ± 12.32 %).

Table 27. Effect of the treatment with SNT on the LVDd, LVDs and EF

Animal group	N	LVDd (mm)	LVDs (mm)	EF (%)
SH-O	10	3.29±0.81	0.91±0.20	96.90±22.2
Model	10	6.24±0.72	6.44±1.59	55.48±12.89
FPS	10	4.45±1.28*	3.81±1.21**	78.03±10.70*
SNT-LD	10	5.37±1.78	4.95±1.95*	69.69±13.91*
SNT-HD	10	4.87±1.47	3.74±1.47**	77.83±12.32*

* $P < 0.05$, ** $P < 0.01$, vs. sham-operated group; values are expressed as mean $\pm$ SD; left ventricular (LV) end-diastolic dimension (LVDd); LV end-systolic dimension (LVDs); ejection fraction (EF) = $\text{LVEDV} - \text{LVESV} / \text{LVEDV}$ (LVEDV = LV end-diastolic volume; LVESV = LV end-systolic volume)

SH-O = sham-operated group (sham-operation, treatment: water)

Model = model group (induction of AMI, treatment: water)

FPS = fosinopril group (induction of AMI, treatment: 900 $\mu\text{g/kg}$ fosinopril)

SNT-LD = SNT-low dosage group (induction of AMI, treatment: 4.5 g/kg SNT)

SNT-HD = SNT-high dosage group (induction of AMI, treatment: 13.5 g/kg SNT)

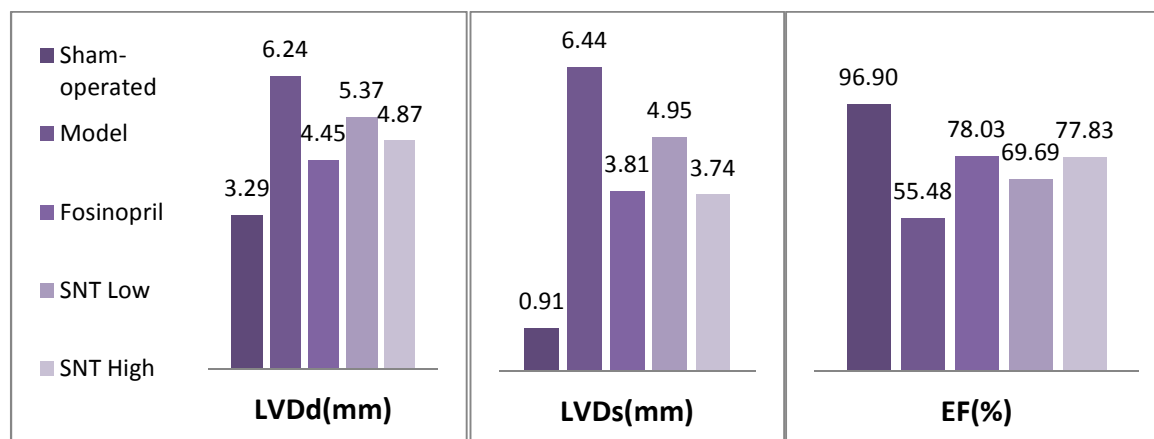


Figure 39. Effect of the treatment with SNT on the LVDd, LVDs and EF

Left ventricular (LV) end-diastolic dimension (LVDd); LV end-systolic dimension (LVDs); ejection fraction (EF) = $\text{LVEDV} - \text{LVESV} / \text{LVEDV}$ (LVEDV = LV end-diastolic volume; LVESV = LV end-systolic volume)

5.2.3 Molecular biological investigations

After echocardiographic measurements, blood samples were taken from the left ventricular (LV) cavity of the anesthetized rats for analysis with enzyme-linked immunosorbent assay (ELISA) tests. Ten serum samples of each group, selected at random, were tested for all the markers listed below. Five of the ten heart tissue samples of each group were randomly picked for the analysis with ELISA. ELISA tests were performed strictly according to the instructions and the protein concentration of each tissue sample was measured with the BCA (bicinchoninic acid) Protein Assay Kit by ELISA.

All results were tested for normal distribution with the aid of the ‘One-Sample Kolmogorov-Smirnov Test’. All analyzed data showed normal distribution. The results were tabulated and presented as the mean $\pm$ standard error of the mean, and the significance of changes was assessed with the aid of one-way repeated measures analysis of variance (ANOVA) followed by Bonferroni’s Holm test for multiple comparisons. The One-way ANOVA Tukey HSD test was used for pairwise multiple comparisons of all six groups and the significance values are listed in tables below each figure. Statistical differences between the Model, FPS, SNT-LD and SNT-HD groups are also pointed out in the figures. The two groups, normal and SH-O groups, served purely as control for the marker selection and operation. A P-value of less than 0.05 was considered to be statistically significant.

The following abbreviations were used in this experiment:

Model = model group (induction of AMI, treatment: water)

FPS = fasinopril group (induction of AMI, treatment: 900 μ g/kg fasinopril)

SNT-LD = SNT-low dosage group (induction of AMI, treatment: 4.5 g/kg SNT)

SNT-HD = SNT-high dosage group (induction of AMI, treatment: 13.5 g/kg SNT)

Normal = blank group (no operation, no treatment)

SH-O = sham-operated group (sham-operation, treatment: water)

Several biomarkers are the prognostic indicators of the development of LV remodeling and heart failure after AMI therefore are conventionally used for the prediction of ventricular remodeling after AMI. In addition, these factors are used to identify potential treatments to reduce

remodeling and counteract the development of heart failure. In order to prove the effects of SNT various markers known to be involved in the process of vascular remodeling were selected and analyzed in serum and heart tissue of rats:

Collagen markers (measured in serum and heart tissue): collagen I, collagen III and transforming growth factor- β 1

Functional index markers (serum): atrial natriuretic peptide and cardiac troponin T

Inflammatory markers (serum): interleukin-6, interleukin- 1β (serum and tissue), tumor necrosis factor- α and high sensitivity C-reactive protein

Vascular active markers (serum): endothelin-1 and nitric oxide

5.2.3.1 Effects of SNT on collagen markers: collagen I, collagen III and transforming growth factor- β 1

An excessive accumulation of collagen is one of the key features of ventricular remodeling²⁷⁰. Excessively fibrous tissue is a sign of both an augmented synthesis of collagen type I (col I) and collagen type III (col III) by cardiac fibroblasts and myofibroblasts and an unaltered or reduced col I and col III degradation by matrix metalloproteinases (MMPs)^{270,271}. Various scientists have determined an elevated level of both col I and col III during cardiac pathologies, the levels being significantly connected to the cardiac fibrosis observed²⁷²⁻²⁷⁴. Thus, serological and tissue level of col I and col III act as a useful tool for determining the status quo of the ongoing pathological process in cardiac tissue after MI and for studying the potential for cardio-protective drugs and strategies. The transforming growth factor- β 1 (TGF- β 1), a cytokine, plays an important role in fibrosis-related cardiovascular disorders, post-angioplasty restenosis and post-infarct ventricular remodeling, all of which can lead to heart failure²⁷⁵. TGF- β 1 serum levels are connected with prognostic values for ventricular remodeling and hypertrophy^{276,277}, and LV function²⁷⁸.

Collagen I in serum and myocardial tissue

The mean (red line) of col I in serum ($\mu\text{g/L}$) of all the groups are presented in figure 40. The mean value of the FPS, SNT-LD and SNT-HD groups were significantly lower than that of the model group (2.67 ± 0.42 , 2.43 ± 0.38 and $2.17 \pm 0.21 \mu\text{g/L}$ vs. $4.11 \pm 0.74 \mu\text{g/L}$) (table 28, for significance see table 29).

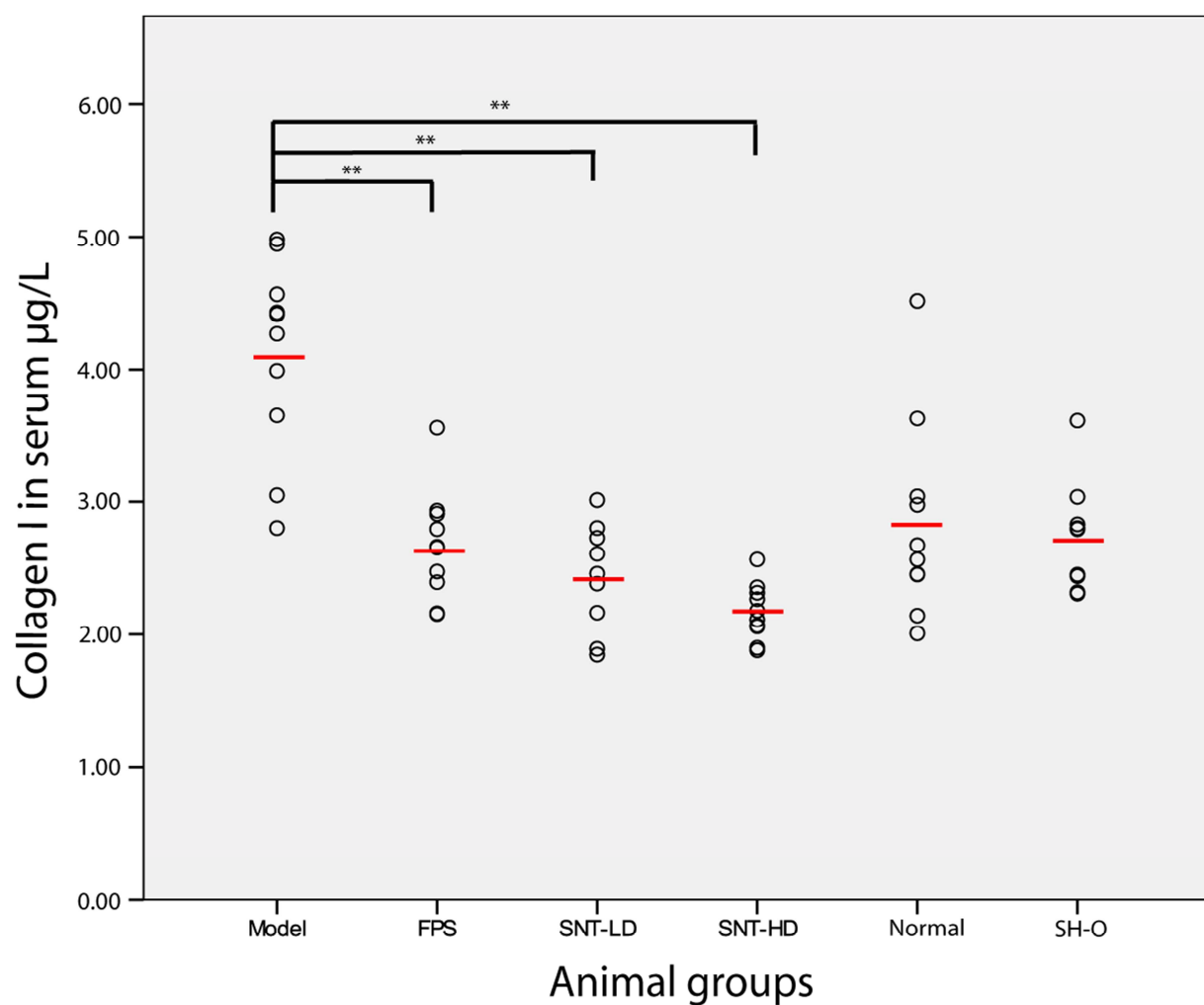


Figure 40. Col I in serum $\mu\text{g/L}$; ** $P < 0.01$; $n = 10$

Table 28. Col I in serum $\mu\text{g/L}$

Animal groups	N	Minimum	Maximum	Mean	Std. Deviation	Variance
Model	10	2.80	4.98	4.11	0.74	0.55
FPS	10	2.15	3.56	2.67	0.42	0.18
SNT-LD	10	1.85	3.01	2.43	0.38	0.15
SNT-HD	10	1.88	2.57	2.17	0.21	0.05
Normal	10	2.01	4.52	2.85	0.75	0.57
SH-O	10	2.31	3.61	2.74	0.40	0.16

n = 10

Table 29. Evaluation of the significant value from multiple group comparison of col I level in serum

Comparison of groups	Significance
Model vs. FPS	0.000
Model vs. SNT-LD	0.000
Model vs. SNT-HD	0.000
Model vs. normal	0.000
Model vs. SH-O	0.000
FPS vs. SNT-LD	0.904
FPS vs. SNT-HD	0.282
FPS vs. normal	0.974
FPS vs. SH-O	1.000
SNT-LD vs. SNT-HD	0.877
SNT-LD vs. normal	0.481
SNT-LD vs. SH-O	0.758
SNT-HD vs. normal	0.058
SNT-HD vs. SH-O	0.157
Normal vs. SH-O	0.998

Numbers in bold = $P < 0.05$

The differences between the mean values of col I in myocardial tissue ($\mu\text{g/g}$) of the rats in the Model, FPS, SNT-LD and SNT-HD groups are shown in figure 41. The mean of all the groups were lower than the model group and a significant difference was observed in the FPS and SNT-HD groups vs. model group (0.86 ± 0.15 and $0.89 \pm 0.14 \mu\text{g/g}$ vs. $1.20 \pm 0.15 \mu\text{g/g}$) (table 30, for significance see table 31).

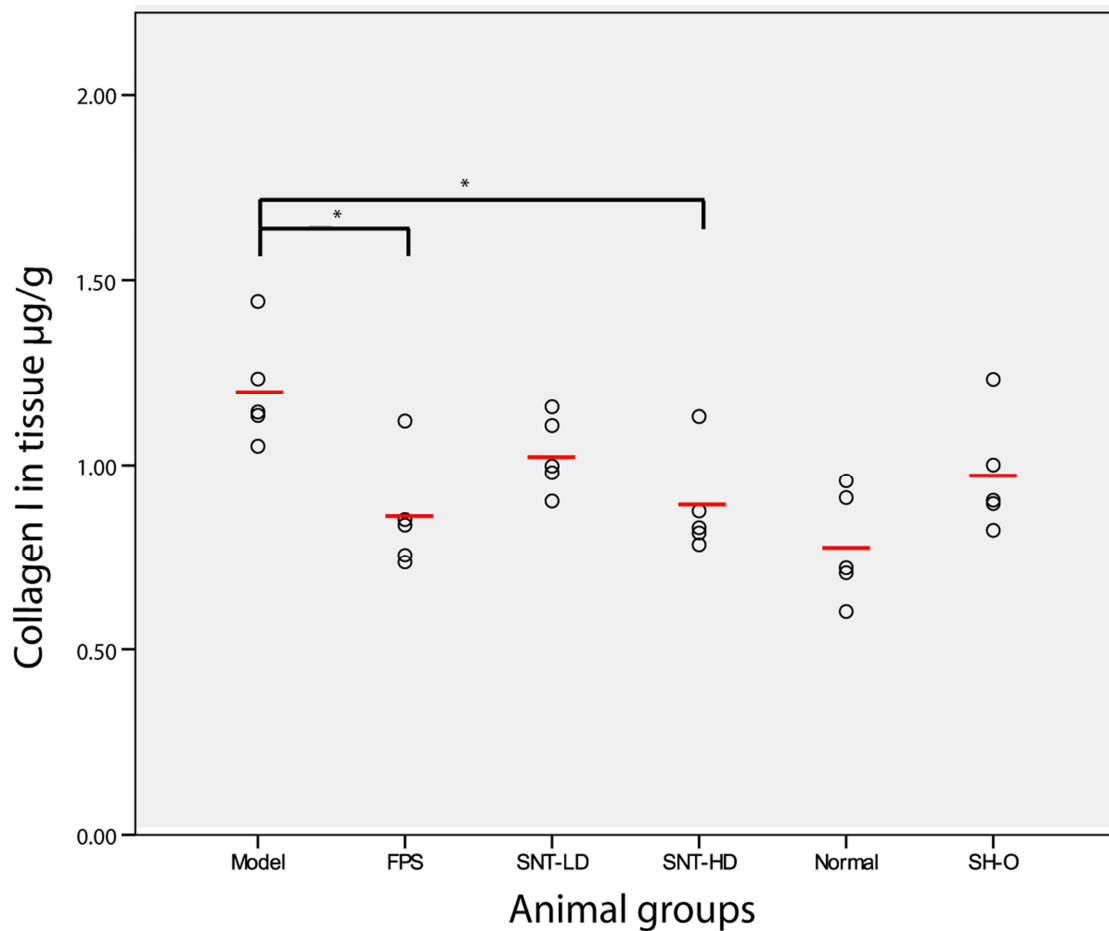


Figure 41. Col I in tissue $\mu\text{g/g}$; * $P < 0.05$; $n = 5$

Table 30. Col I in tissue $\mu\text{g/g}$

Animal groups	N	Minimum	Maximum	Mean	Std. Deviation	Variance
Model	5	1.05	1.44	1.20	0.15	0.02
FPS	5	0.74	1.12	0.86	0.15	0.02
SNT-LD	5	0.90	1.16	1.03	0.10	0.01
SNT-HD	5	0.79	1.13	0.89	0.14	0.02
Normal	5	0.60	0.96	0.78	0.15	0.02
SH-O	5	0.82	1.23	0.97	0.16	0.03

n = 5

Table 31. Evaluation of the significant value from multiple group comparison of col I level in tissue

Comparison of groups	Significance
Model vs. FPS	0.011
Model vs. SNT-LD	0.428
Model vs. SNT-HD	0.022
Model vs. normal	0.001
Model vs. SH-O	0.155
FPS vs. SNT-LD	0.449
FPS vs. SNT-HD	1.000
FPS vs. normal	0.947
FPS vs. SH-O	0.819
SNT-LD vs. SNT-HD	0.633
SNT-LD vs. normal	0.103
SNT-LD vs. SH-O	0.987
SNT-HD vs. normal	0.840
SNT-HD vs. SH-O	0.936
Normal vs. SH-O	0.316

Numbers in bold = $P < 0.05$

Collagen III in serum and myocardial tissue

The differences between the mean values of col III in serum ($\mu\text{g/L}$) of the model group and the three treated groups were significantly lower and are presented in figure 42 and table 32 and 33 ($2.68 \pm 0.43 \mu\text{g/L}$ vs. FPS 2.00 ± 0.45 , SNT-LD 2.02 ± 0.47 and SNT-HD $1.96 \pm 0.47 \mu\text{g/L}$).

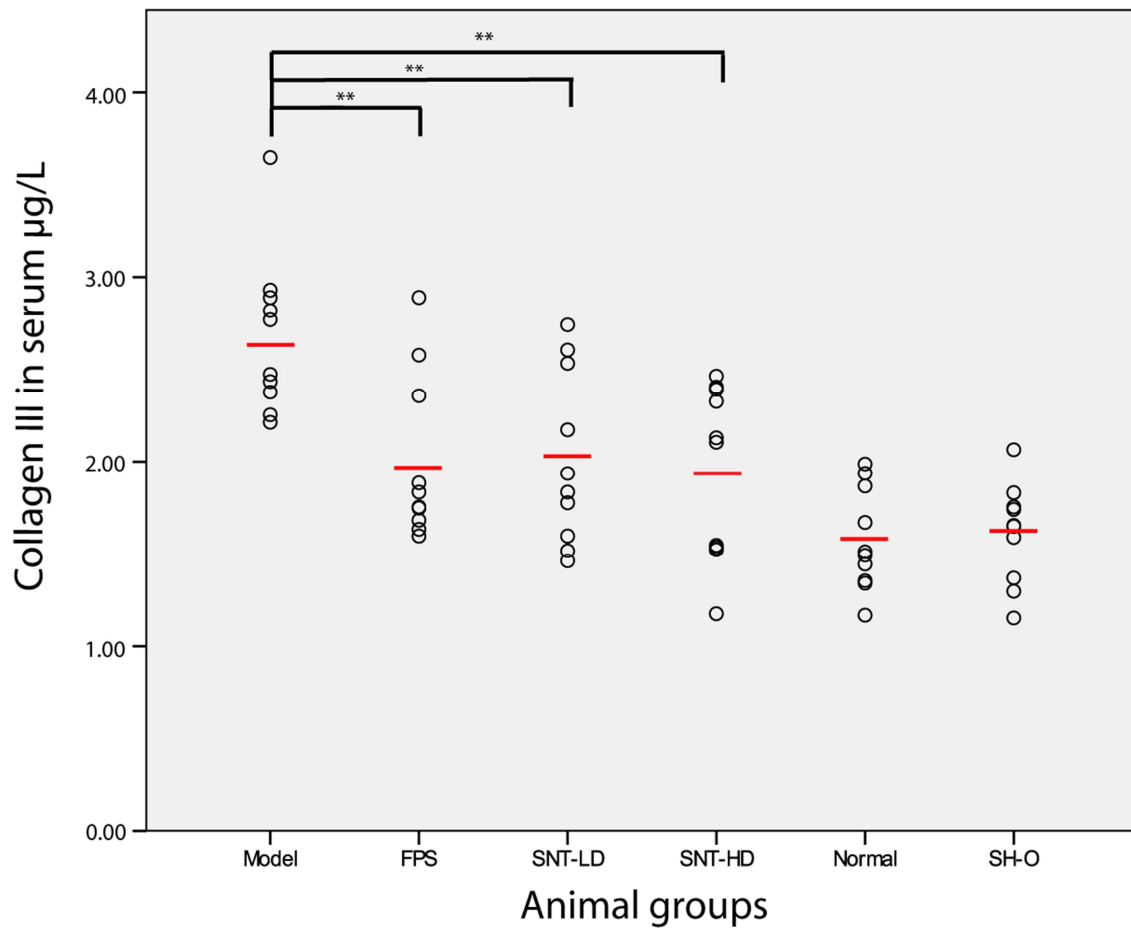


Figure 42. Col III in serum $\mu\text{g/L}$; ** $P < 0.01$; $n = 10$

Table 32. Col III in serum $\mu\text{g/L}$

Animal groups	N	Minimum	Maximum	Mean	Std. Deviation	Variance
Model	10	2.21	3.65	2.68	0.43	0.18
FPS	10	1.60	2.89	2.00	0.45	0.20
SNT-LD	10	1.47	2.74	2.02	0.47	0.22
SNT-HD	10	1.18	2.46	1.96	0.47	0.22
Normal	10	1.17	1.99	1.58	0.28	0.08
SH-O	10	1.16	2.07	1.61	0.27	0.07

n = 10

Table 33. Evaluation of the significant value from multiple group comparison of col III level in serum

Comparison of groups	Significance
Model vs. FPS	0.005
Model vs. SNT-LD	0.007
Model vs. SNT-HD	0.003
Model vs. normal	0.000
Model vs. SH-O	0.000
FPS vs. SNT-LD	1.000
FPS vs. SNT-HD	1.000
FPS vs. normal	0.205
FPS vs. SH-O	0.286
SNT-LD vs. SNT-HD	1.000
SNT-LD vs. normal	0.162
SNT-LD vs. SH-O	0.230
SNT-HD vs. normal	0.292
SNT-HD vs. SH-O	0.391
Normal vs. SH-O	1.000

Numbers in bold = $P < 0.05$

The differences between the mean values of col III in cardiac tissue ($\mu\text{g/g}$) of the rats in the model, FPS, SNT-LD and SNT-HD groups are shown in figure 43 and table 34 and 35. The mean of all the groups were lower than the model group and a significant difference was seen in the FPS and SNT-HD groups vs. the model group (0.33 ± 0.04 and $0.30 \pm 0.04 \mu\text{g/g}$ vs. $0.45 \pm 0.04 \mu\text{g/g}$).

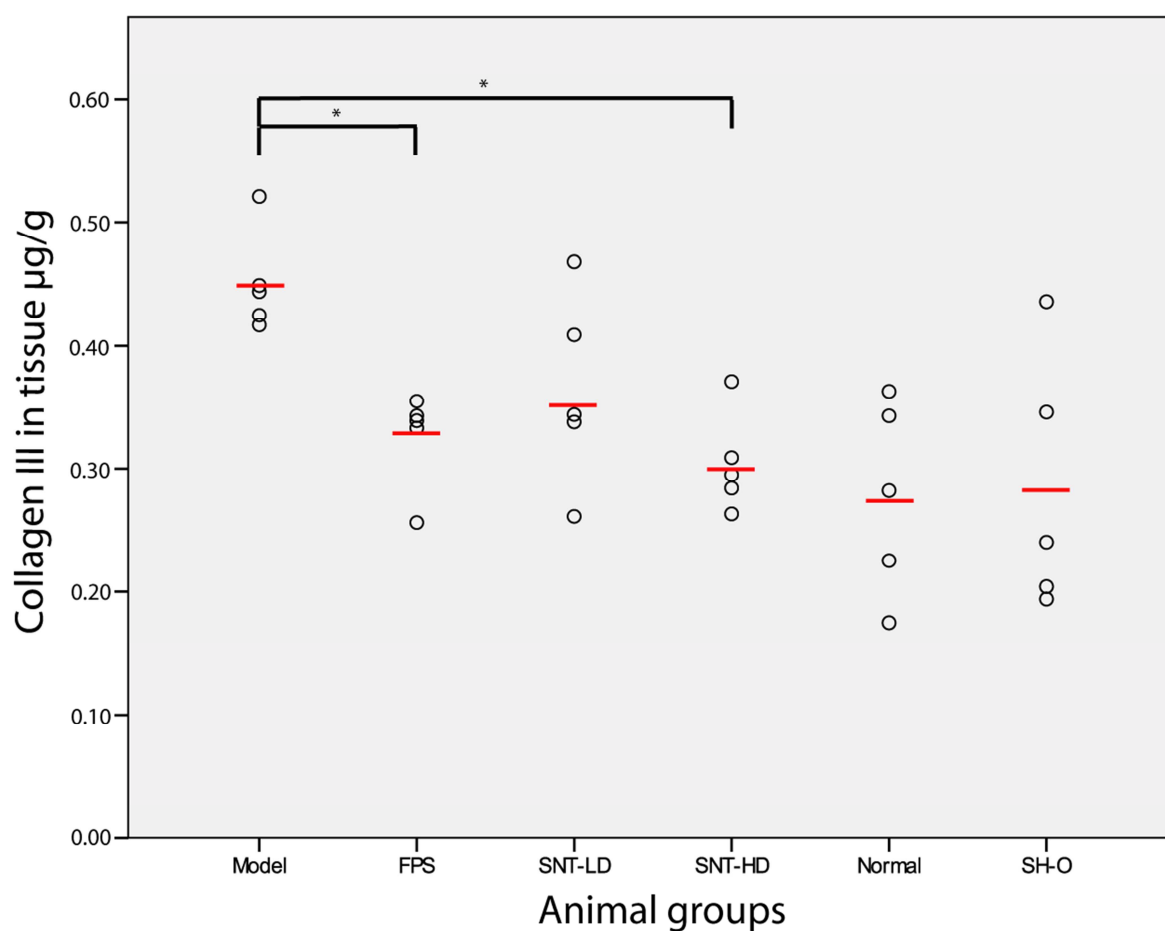


Figure 43. Col III in tissue $\mu\text{g/g}$; * $P < 0.05$; $n = 5$

Table 34. Col III in tissue $\mu\text{g/g}$

Animal groups	N	Minimum	Maximum	Mean	Std. Deviation	Variance
Model	5	0.42	0.52	0.45	0.04	0.00
FPS	5	0.26	0.36	0.33	0.04	0.00
SNT-LD	5	0.26	0.47	0.36	0.08	0.01
SNT-HD	5	0.26	0.37	0.30	0.04	0.00
Normal	5	0.18	0.36	0.28	0.08	0.01
SH-O	5	0.19	0.44	0.28	0.10	0.01

n = 5

Table 35. Evaluation of the significant value from multiple group comparison of col III level in tissue

Comparison of groups	Significance
Model vs. FPS	0.043
Model vs. SNT-LD	0.364
Model vs. SNT-HD	0.026
Model vs. normal	0.006
Model vs. SH-O	0.009
FPS vs. SNT-LD	0.944
FPS vs. SNT-HD	0.996
FPS vs. normal	0.877
FPS vs. SH-O	0.929
SNT-LD vs. SNT-HD	0.740
SNT-LD vs. normal	0.374
SNT-LD vs. SH-O	0.456
SNT-HD vs. normal	0.989
SNT-HD vs. SH-O	0.997
Normal vs. SH-O	1.000

Numbers in bold $P < 0.05$

Transforming growth factor- β 1 in serum and myocardial tissue

There were no differences between the mean values of TGF- β 1 in serum (ng/L) in the four groups compared (figure 44 and table 36 and 37).

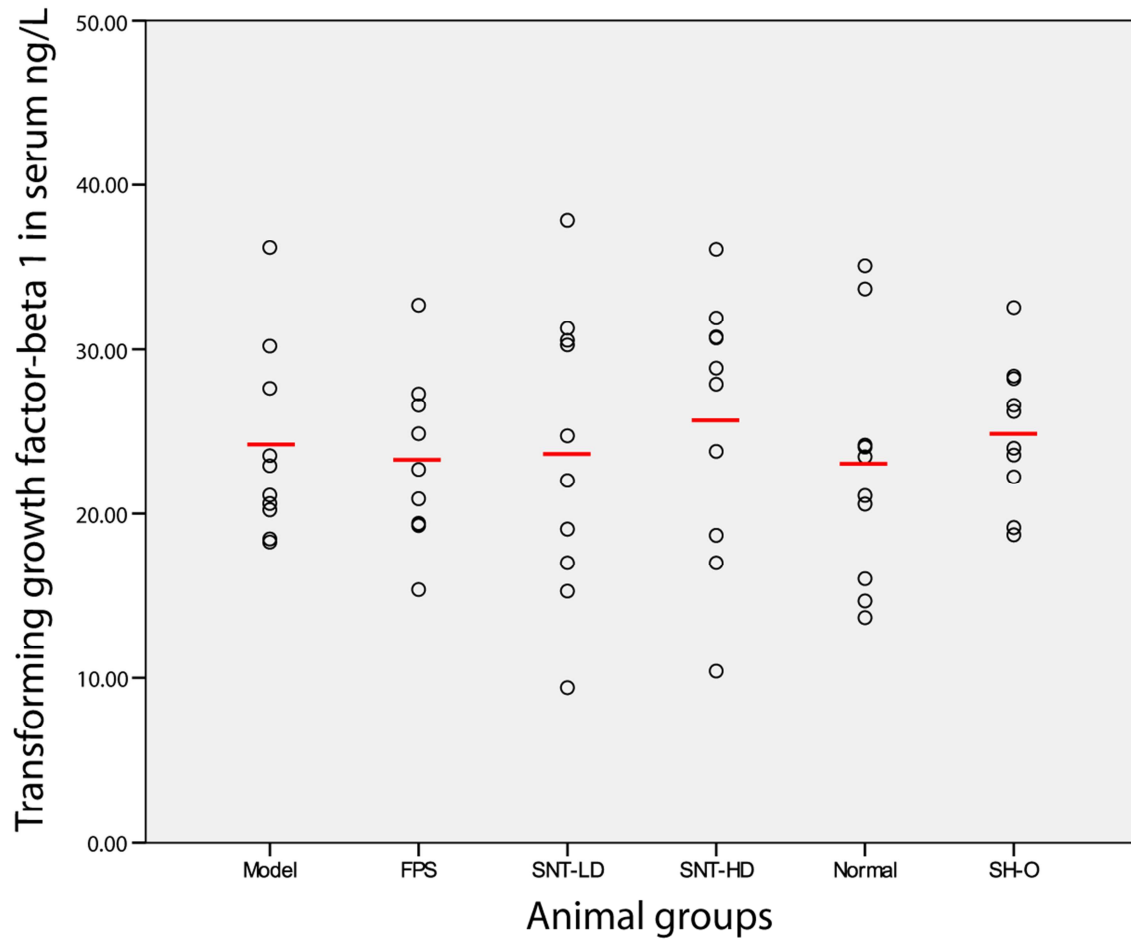


Figure 44. TGF- β 1 in serum ng/L; $n = 10$

Table 36. *TGF- β 1* in serum ng/L

Animal groups	N	Minimum	Maximum	Mean	Std. Deviation	Variance
Model	10	18.28	36.19	23.91	5.76	33.23
FPS	10	15.39	32.70	22.84	5.07	25.71
SNT-LD	10	9.41	37.88	23.75	8.78	77.04
SNT-HD	10	10.41	36.08	25.61	7.98	63.70
Normal	10	13.68	35.09	22.65	7.27	52.81
SH-O	10	18.72	32.56	24.95	4.31	18.59

n =10

Table 37. *Evaluation of the significant value from multiple group comparison of TGF- β 1 level in serum*

Comparison of groups	Significance
Model vs. FPS	0.999
Model vs. SNT-LD	1.000
Model vs. SNT-HD	0.993
Model vs. normal	0.998
Model vs. SH-O	0.999
FPS vs. SNT-LD	1.000
FPS vs. SNT-HD	0.939
FPS vs. normal	1.000
FPS vs. SH-O	0.981
SNT-LD vs. SNT-HD	0.989
SNT-LD vs. normal	0.999
SNT-LD vs. SH-O	0.999
SNT-HD vs. normal	0.921
SNT-HD vs. SH-O	1.000
Normal vs. SH-O	0.972

The mean value of TGF- β 1 in myocardial tissue (ng/g) was lower in the FPS, SNT-LD and SNT-HD groups than in the model group but no significant difference was observed (figure 45, table 38 and 39). However, the mean value of the Normal group (6.33 ± 2.55 ng/g) was very similar to that of the model group (6.48 ± 1.79 ng/g).

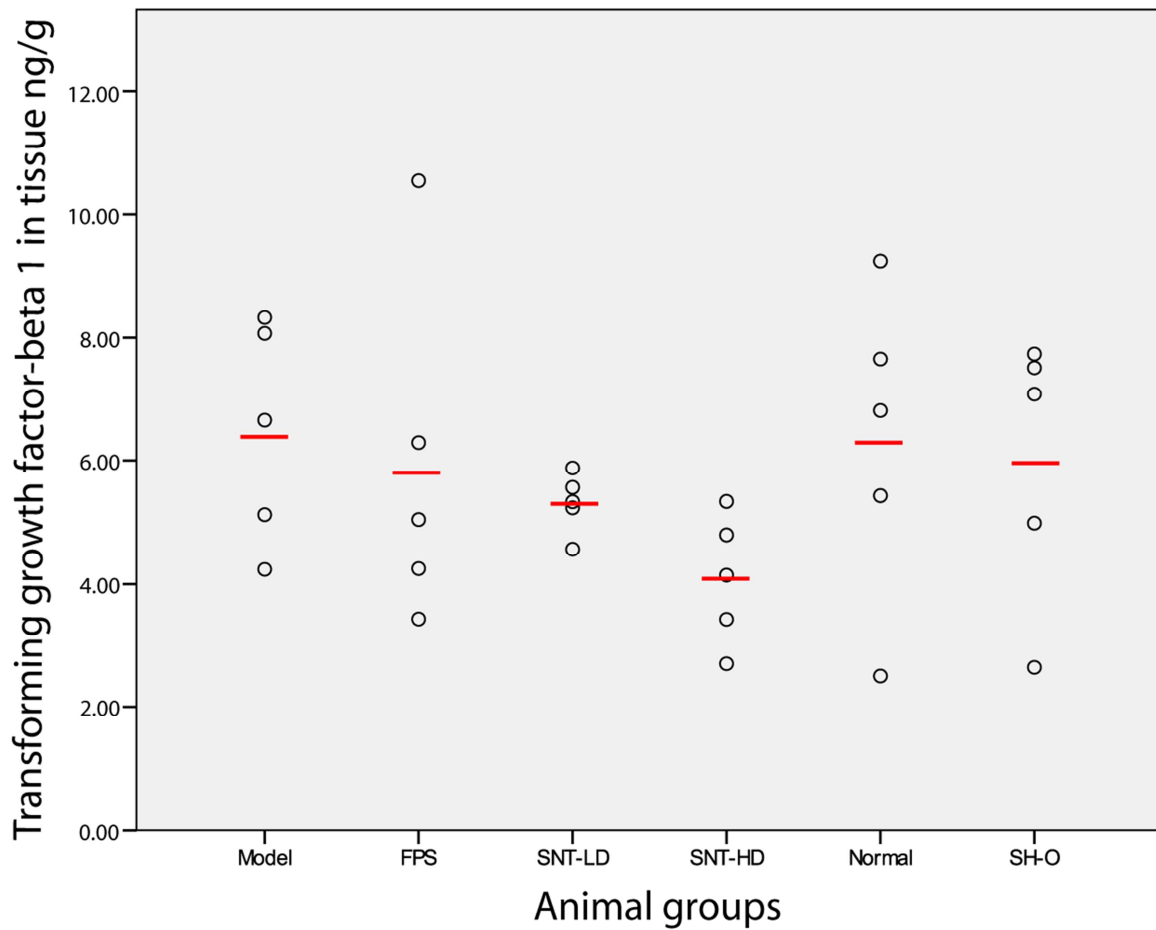


Figure 45. TGF- β 1 in tissue ng/g; $n = 5$

Table 38. *TGF- β 1 in tissue ng/g*

Animal groups	N	Minimum	Maximum	Mean	Std. Deviation	Variance
Model	5	4.24	8.33	6.48	1.79	3.21
FPS	5	3.43	10.55	5.91	2.80	7.82
SNT-LD	5	4.57	5.88	5.32	0.49	0.24
SNT-HD	5	2.70	5.34	4.08	1.05	1.11
Normal	5	2.50	9.24	6.33	2.55	6.48
SH-O	5	2.64	7.74	5.99	2.17	4.69

n = 5

Table 39. *Evaluation of the significant value from multiple group comparison of TGF- β 1 level in tissue*

Comparison of groups	Significance
Model vs. FPS	0.997
Model vs. SNT-LD	0.934
Model vs. SNT-HD	0.416
Model vs. normal	1.000
Model vs. SH-O	0.999
FPS vs. SNT-LD	0.997
FPS vs. SNT-HD	0.690
FPS vs. normal	0.999
FPS vs. SH-O	1.000
SNT-LD vs. SNT-HD	0.918
SNT-LD vs. normal	0.963
SNT-LD vs. SH-O	0.994
SNT-HD vs. normal	0.489
SNT-HD vs. SH-O	0.653
Normal vs. SH-O	1.000

5.2.3.2 Effects of SNT on functional index markers: atrial natriuretic peptide and cardiac troponin T

Atrial natriuretic peptide (ANP) is a vasodilator which is released in response to increasing atrial filling pressures²⁷⁹. As a response to wall stress, atrial natriuretic peptide reduces cardiac preload and afterload, regulates the blood pressure by augmenting microvascular permeability²⁸⁰ and induces the relaxation of vascular smooth muscle cells^{281,282}. The regulation of blood pressure is also maintained by ANP acting on the renin-angiotensin-aldosterone system²⁸³. In clinical practice, ANP plays an important role as a biomarker of heart failure because it is proved to be highly effective for the diagnosis and risk-stratification due to its consistent rise in the plasma level in hypertrophy and heart failure²⁸². Cardiac troponin T (cTnT), located along with tropomyosin on the actin filament, is essential for the regulation of calcium-mediated contraction of the skeletal and cardiac muscles²⁸⁴. An increase of the cTnT level is notable with increased myocardial wall stress and LV end-diastolic pressure^{285,286}. cTnT is suggested as a first-line biomarker for the diagnosis of acute myocardial infarction by the Joint European Society of Cardiology-American College of Cardiology Foundation-American Heart Association-World Heart Federation Task Force²⁸⁶. Persistently augmented cTnT levels have been observed during continuous cardiac remodeling, concluding that this marker indicates an ongoing myocyte injury^{284,287,288}.

Atrial natriuretic peptide in serum

The determined ANP levels in the serum (ng/L) were very similar in all the groups (figure 46, table 40 and 41). Whereas SNT-LD and SNT-HD groups showed the highest mean which were 397.63 ± 80.19 ng/L and 407.20 ± 49.96 ng/L, but no significant difference was observed.

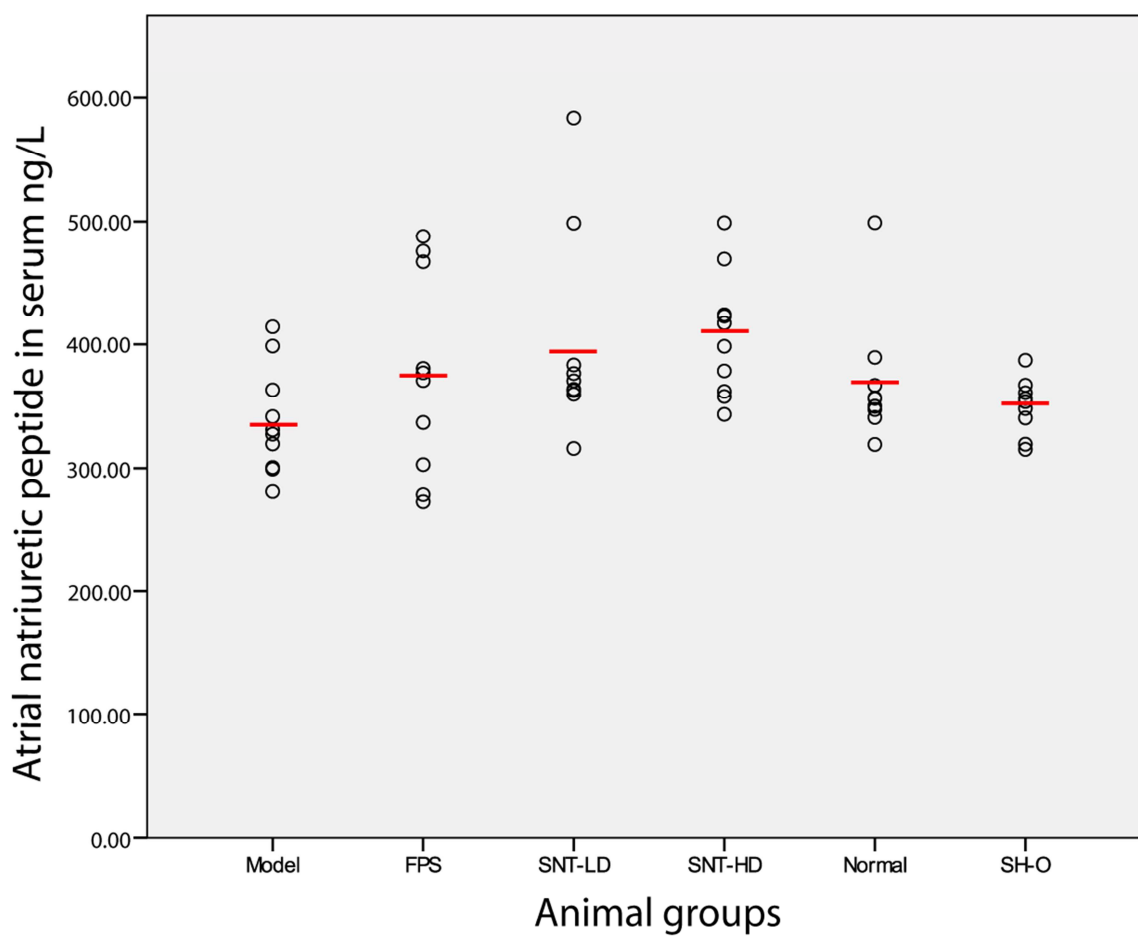


Figure 46. ANP in serum ng/L; n= 10

Table 40. ANP in serum ng/L

Animal groups	N	Minimum	Maximum	Mean	Std. Deviation	Variance
Model	10	280.84	414.29	337.57	43.20	1865.93
FPS	10	272.63	487.93	374.93	80.25	6440.78
SNT-LD	10	315.84	583.43	397.63	80.19	6430.65
SNT-HD	10	343.16	498.94	407.20	49.96	2495.55
Normal	10	319.01	499.03	367.34	49.62	2462.42
SH-O	10	315.12	387.06	350.00	21.32	454.57

n = 10

Table 41. Evaluation of the significant value from multiple group comparison of ANP level in serum

Comparison of groups	Significance
Model vs. FPS	0.702
Model vs. SNT-LD	0.205
Model vs. SNT-HD	0.095
Model vs. normal	0.859
Model vs. SH-O	0.997
FPS vs. SNT-LD	0.951
FPS vs. SNT-HD	0.813
FPS vs. normal	1.000
FPS vs. SH-O	0.928
SNT-LD vs. SNT-HD	0.999
SNT-LD vs. normal	0.850
SNT-LD vs. SH-O	0.451
SNT-HD vs. normal	0.642
SNT-HD vs. SH-O	0.251
Normal vs. SH-O	0.985

Cardiac troponin T in serum

The mean values of cTnT in serum ($\mu\text{g/L}$) of all groups are presented in figure 47 and table 42 and the significance in table 43. The mean of the FPS, SNT-LD and SNT-HD groups were lower than that of the model group, although not significantly. The determined levels of normal and SH-O groups were remarkably lower than those of the other groups ($0.02 - 0.03 \mu\text{g/L}$ vs. $0.16 - 0.24 \mu\text{g/L}$).

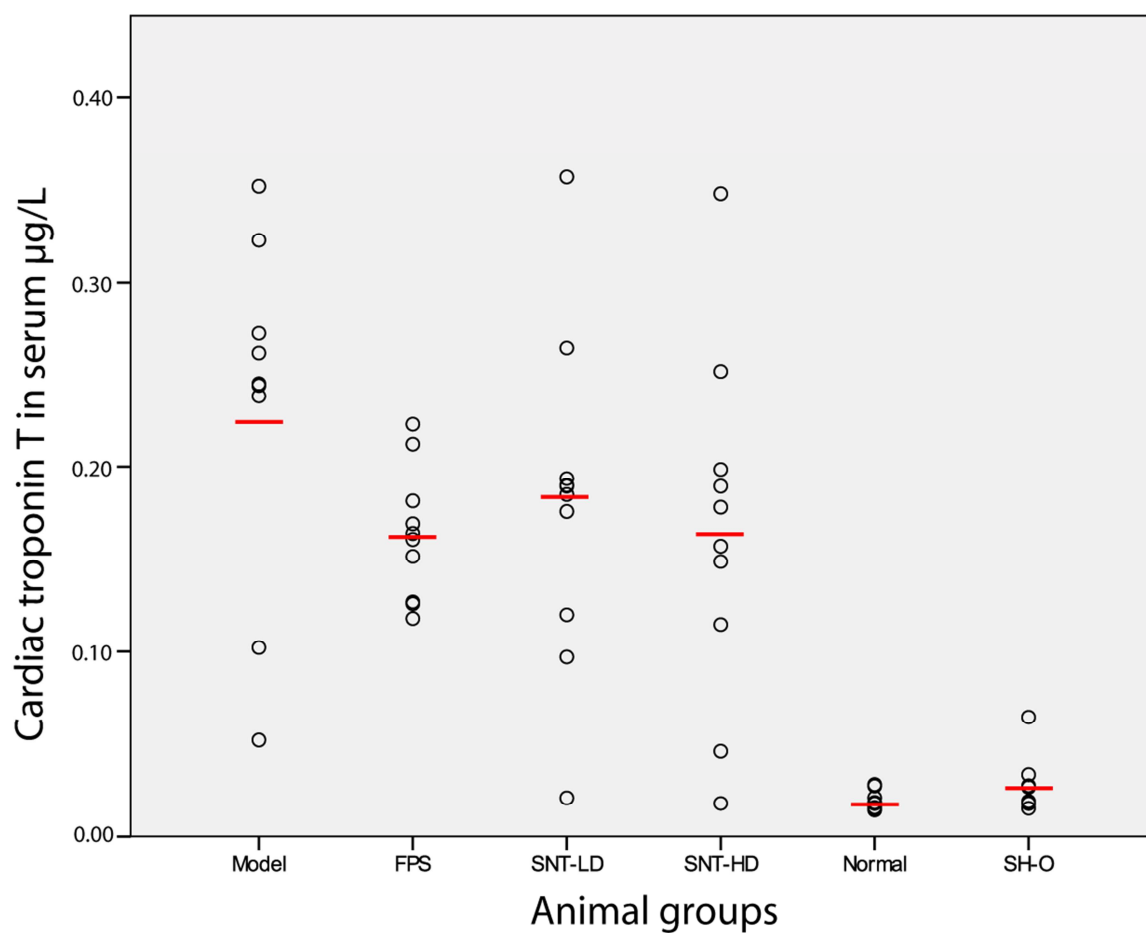


Figure 47. CTnT in serum $\mu\text{g/L}$; $n = 10$

Table 42. CTnT in serum $\mu\text{g/L}$

Animal groups	N	Minimum	Maximum	Mean	Std. Deviation	Variance
Model	10	0.05	0.35	0.24	0.10	0.01
FPS	10	0.12	0.22	0.16	0.04	0.00
SNT-LD	10	0.02	0.36	0.18	0.09	0.01
SNT-HD	10	0.02	0.35	0.17	0.10	0.01
Normal	10	0.01	0.03	0.02	0.01	0.00
SH-O	10	0.02	0.06	0.03	0.01	0.00

n = 10

Table 43. Evaluation of the significant value from multiple group comparison of cTnT level in serum

Comparison of groups	Significance
Model vs. FPS	0.109
Model vs. SNT-LD	0.300
Model vs. SNT-HD	0.123
Model vs. normal	0.000
Model vs. SH-O	0.000
FPS vs. SNT-LD	0.995
FPS vs. SNT-HD	1.000
FPS vs. normal	0.000
FPS vs. SH-O	0.001
SNT-LD vs. SNT-HD	0.997
SNT-LD vs. normal	0.000
SNT-LD vs. SH-O	0.000
SNT-HD vs. normal	0.000
SNT-HD vs. SH-O	0.001
Normal vs. SH-O	1.000

Numbers in bold = $P < 0.05$

5.2.3.3 Effects of SNT on inflammatory markers: interleukin-6, interleukin-1 β , tumor necrosis factor- α , high sensitivity C-reactive protein

Mechanical stress is associated with acute phase response, and therefore a trigger for the up-regulation and myocardial production of the main regulators interleukin-6 (IL-6), tumor necrosis factor- α (TNF- α) and interleukin-1 β (IL-1 β) in the infarcted and non-infarcted region of the heart²⁸⁹, which are not normally expressed in a healthy heart^{289,290}. Among the cytokines, IL-6, TNF- α and IL-1 β are those most frequently attributed to the ventricular remodeling post-infarction²⁸⁹. Various activities are attributed to the elevated expression of IL-6, TNF- α and IL-1 β such as an augmentation of matrix metalloproteinase (MMP) activity, an increase in expression of collagen in the non-infarcted myocardium, the regulation of integrins and angiogenesis, an increase in natriuretic peptides²⁸⁹, an induction of myocyte intercellular adhesion molecule-1 (ICAM-1)²⁹¹, an activation of neutral sphingomyelinase and nitric oxide production^{269,292} and induction of the synthesis of C-reactive protein^{293,294}. High-sensitivity C-reactive protein (hs-CRP) increases if any infection, tissue injury or inflammation occurs²⁹⁵. Thus hs-CRP is commonly used as a major predictor of ventricular remodeling due to its elevated peak serum level after MI²⁹⁶. Altered levels of hs-CRP are associated with the risk of developing cardiovascular disease²⁹⁷. Sustained activation of hs-CRP may be connected to an enhancement of myocardial damage and dysfunction. Hs-CRP activates the local complement pathway by binding the extracellular matrix components²⁹⁸. It is believed that hs-CRP accounts for both increasing the inflammatory tissue damage and the host defense against infection due to its binding to and precipitating soluble ligands and aggregating individual ligands²⁹⁵. These pro-inflammatory cytokines can act in both ways, either resulting in healing and restoration of heart function, or resulting in acute cardiac rupture or chronic dilatation²⁸⁹. These markers have been recognized as mediators for pathological ventricular remodeling, with increasing evidence linking these molecules to the progress of heart failure²⁹⁹.

Interleukin-6 in serum

The differences between the mean values of IL-6 in serum (ng/L) of the various groups are presented in figure 48 and table 44. All groups showed lower levels of IL-6 than those of the model group, although not significantly lower (table 45).

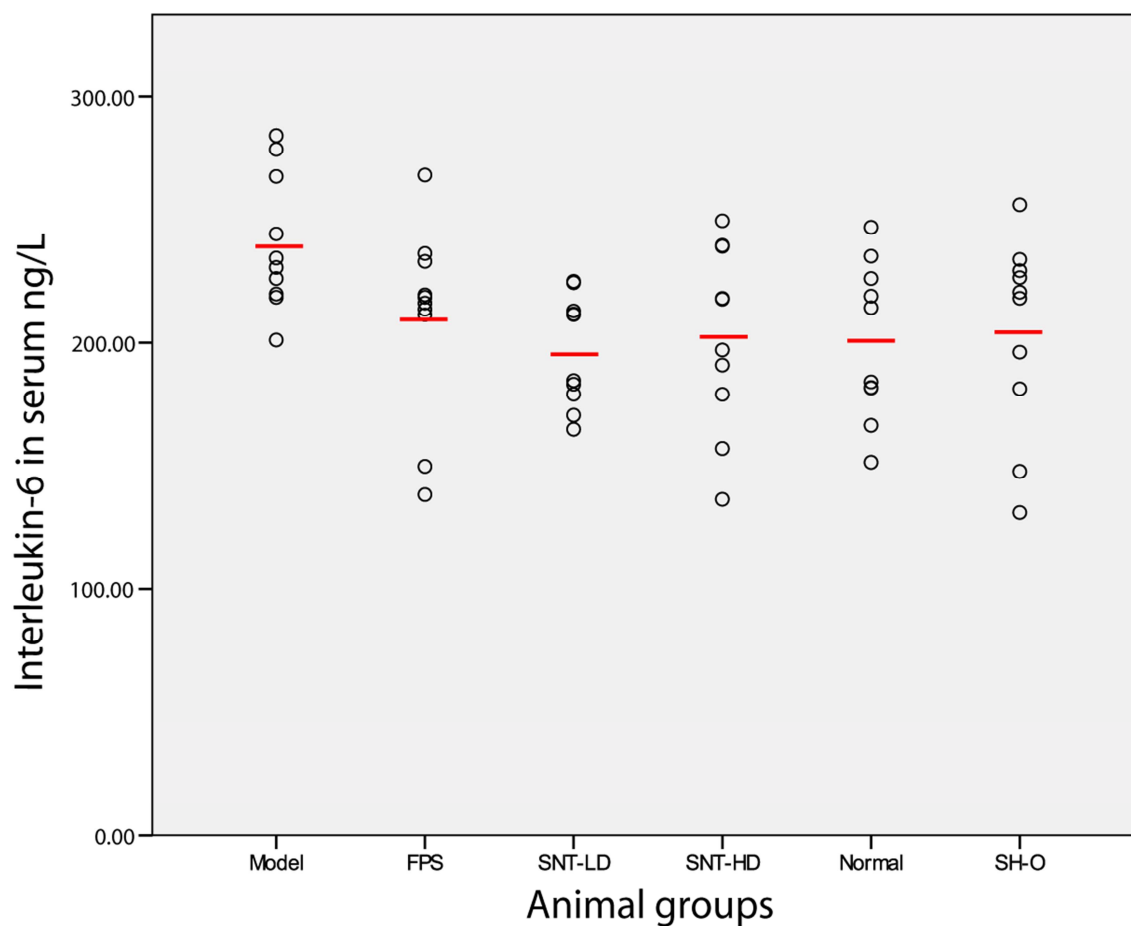


Figure 48. IL-6 in serum ng/L; $n = 10$

Table 44. IL-6 in serum ng/L

Animal groups	N	Minimum	Maximum	Mean	Std. Deviation	Variance
Model	10	201.12	284.42	240.65	27.80	772.61
FPS	10	138.48	268.37	210.60	38.83	1507.98
SNT-LD	10	164.87	225.06	196.81	22.68	514.36
SNT-HD	10	136.58	249.75	202.55	37.37	1396.69
Normal	10	151.67	247.13	200.76	31.80	1011.05
SH-O	10	131.21	256.27	204.17	39.83	1586.69

n = 10

Table 45. Evaluation of the significant value from multiple group comparison of IL-6 level in serum

Comparison of groups	Significance
Model vs. FPS	0.357
Model vs. SNT-LD	0.055
Model vs. SNT-HD	0.133
Model vs. normal	0.102
Model vs. SH-O	0.166
FPS vs. SNT-LD	0.941
FPS vs. SNT-HD	0.994
FPS vs. normal	0.986
FPS vs. SH-O	0.998
SNT-LD vs. SNT-HD	0.999
SNT-LD vs. normal	1.000
SNT-LD vs. SH-O	0.996
SNT-HD vs. normal	1.000
SNT-HD vs. SH-O	1.000
Normal vs. SH-O	1.000

Interleukin-1 β in serum and myocardial tissue

The mean values of IL-1 β in the serum (ng/L) of all groups are presented in figure 49. A significant difference was observed between the model group and the three treated groups (FPS, SNT-LD and SNT-HD group) which all showed lower levels of IL-1 β than the model group (151.17 ± 16.62 , 170.91 ± 15.09 , 170.81 ± 23.30 ng/L vs. 199.85 ± 2.54 ng/L) (table 46, for significance see table 47).

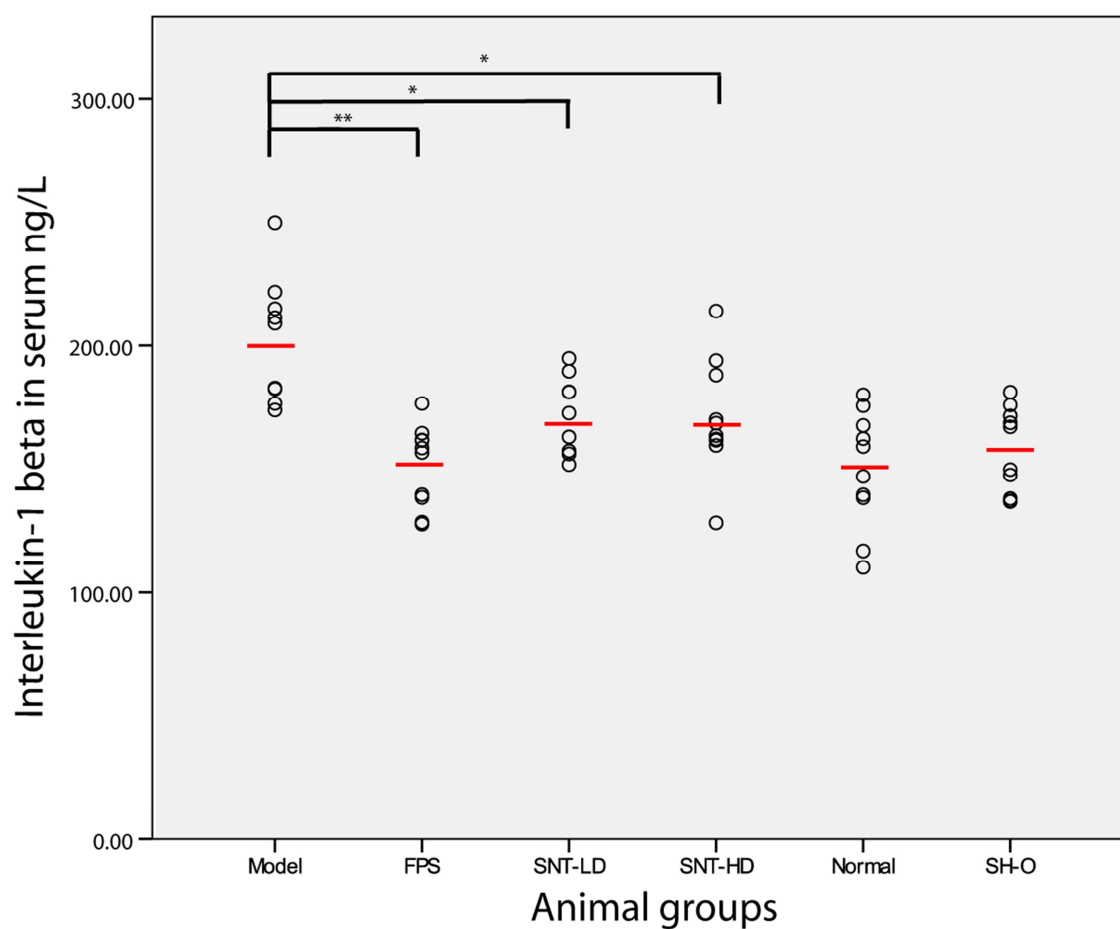


Figure 49. IL-1 β in serum ng/L; * $P < 0.05$, ** $P < 0.01$; $n = 10$

Table 46. *IL-1 β in serum ng/L*

Animal groups	N	Minimum	Maximum	Mean	Std. Deviation	Variance
Model	10	173.70	249.98	199.85	25.44	647.36
FPS	10	127.56	176.37	151.17	16.62	276.06
SNT-LD	10	151.52	194.76	170.91	15.09	227.72
SNT-HD	10	128.03	214.04	170.81	23.30	543.02
Normal	10	110.10	179.93	149.48	23.65	559.50
SH-O	10	136.58	180.99	157.15	17.34	300.57

n = 10

Table 47. *Evaluation of the significant value from multiple group comparison of IL-1 β level in serum*

Comparison of groups	Significance
Model vs. FPS	0.000
Model vs. SNT-LD	0.031
Model vs. SNT-HD	0.030
Model vs. normal	0.000
Model vs. SH-O	0.000
FPS vs. SNT-LD	0.283
FPS vs. SNT-HD	0.289
FPS vs. normal	1.000
FPS vs. SH-O	0.987
SNT-LD vs. SNT-HD	1.000
SNT-LD vs. normal	0.203
SNT-LD vs. SH-O	0.672
SNT-HD vs. normal	0.207
SNT-HD vs. SH-O	0.679
Normal vs. SH-O	0.960

Numbers in bold = P < 0.05

The differences between the mean values of IL-1 β in cardiac tissue (ng/g) of the rats in the Model, FPS, SNT-LD and SNT-HD groups are shown in figure 50 and table 48. The mean of all the groups were lower than those of the model group and a significant difference was observed in the FPS and SNT-HD groups vs. the model group (table 49). The mean of the SNT-HD group (30.37 ± 1.91 ng/L) was very similar to that of the normal and SH-O groups (29.68 ± 5.63 and 29.73 ± 1.53 ng/L).

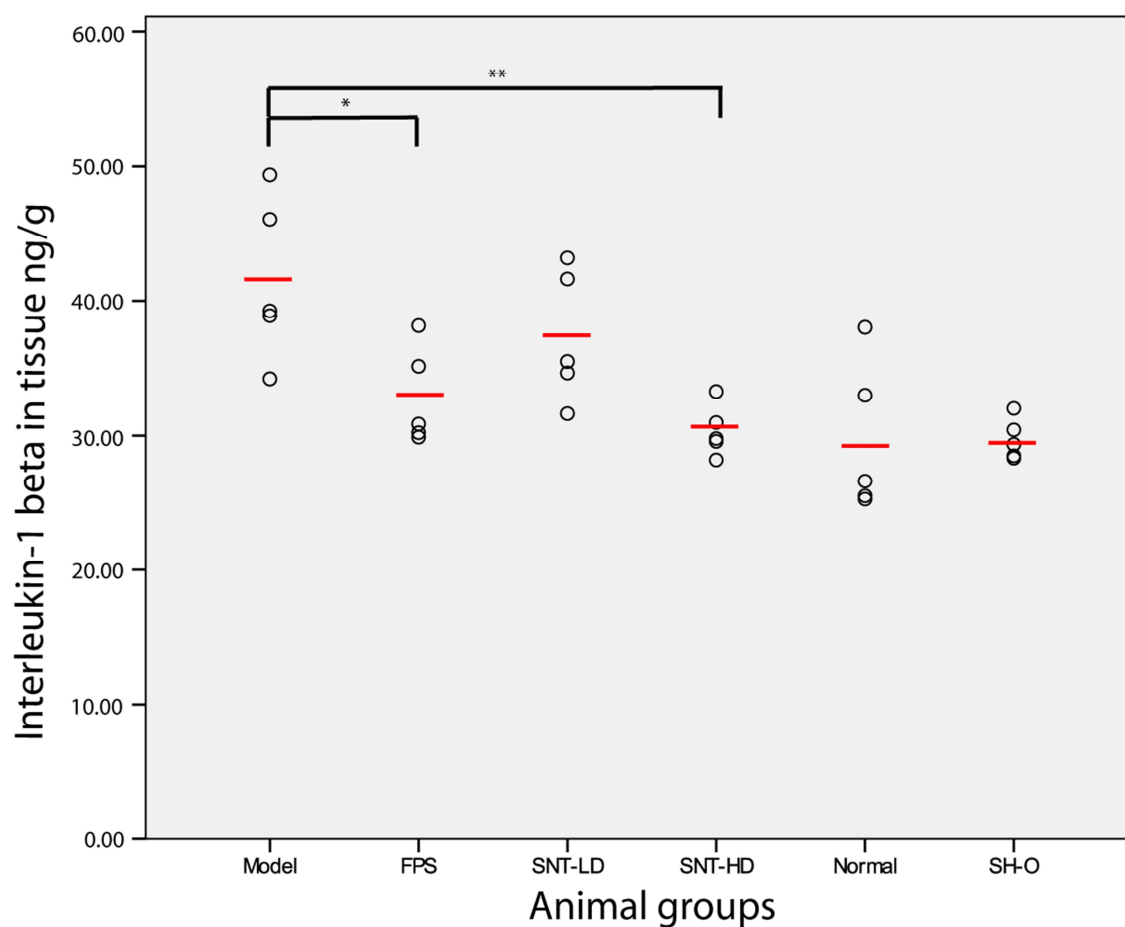


Figure 50. IL-1 β in tissue ng/g; * $P < 0.05$, ** $P < 0.01$; $n = 5$

Table 48. *IL-1 β* in tissue ng/g

Animal groups	N	Minimum	Maximum	Mean	Std. Deviation	Variance
Model	5	34.22	49.37	41.56	6.08	36.95
FPS	5	29.89	38.14	32.85	3.64	13.22
SNT-LD	5	31.64	43.19	37.32	4.89	23.89
SNT-HD	5	28.20	33.29	30.37	1.91	3.65
Normal	5	25.27	38.02	29.68	5.63	31.74
SH-O	5	28.33	32.03	29.73	1.53	2.35

n = 5

Table 49. Evaluation of the significant value from multiple group comparison of *IL-1 β* level in tissue

Comparison of groups	Significance
Model vs. FPS	0.041
Model vs. SNT-LD	0.636
Model vs. SNT-HD	0.005
Model vs. normal	0.003
Model vs. SH-O	0.003
FPS vs. SNT-LD	0.584
FPS vs. SNT-HD	0.940
FPS vs. normal	0.850
FPS vs. SH-O	0.858
SNT-LD vs. SNT-HD	0.150
SNT-LD vs. normal	0.092
SNT-LD vs. SH-O	0.096
SNT-HD vs. normal	1.000
SNT-HD vs. SH-O	1.000
Normal vs. SH-O	1.000

Numbers in bold = P < 0.05

Tumor necrosis factor- α in serum

The mean level of TNF- α in serum (ng/L) was lower in the treated groups, normal and SH-O groups than in the model group (figure 51, table 50). The mean values of FPS and SNT-HD groups showed a significant difference vs. the model group (22.17 ± 8.66 and 23.94 ± 4.27 ng/L vs. 33.23 ± 5.78 ng/L) (table 51).

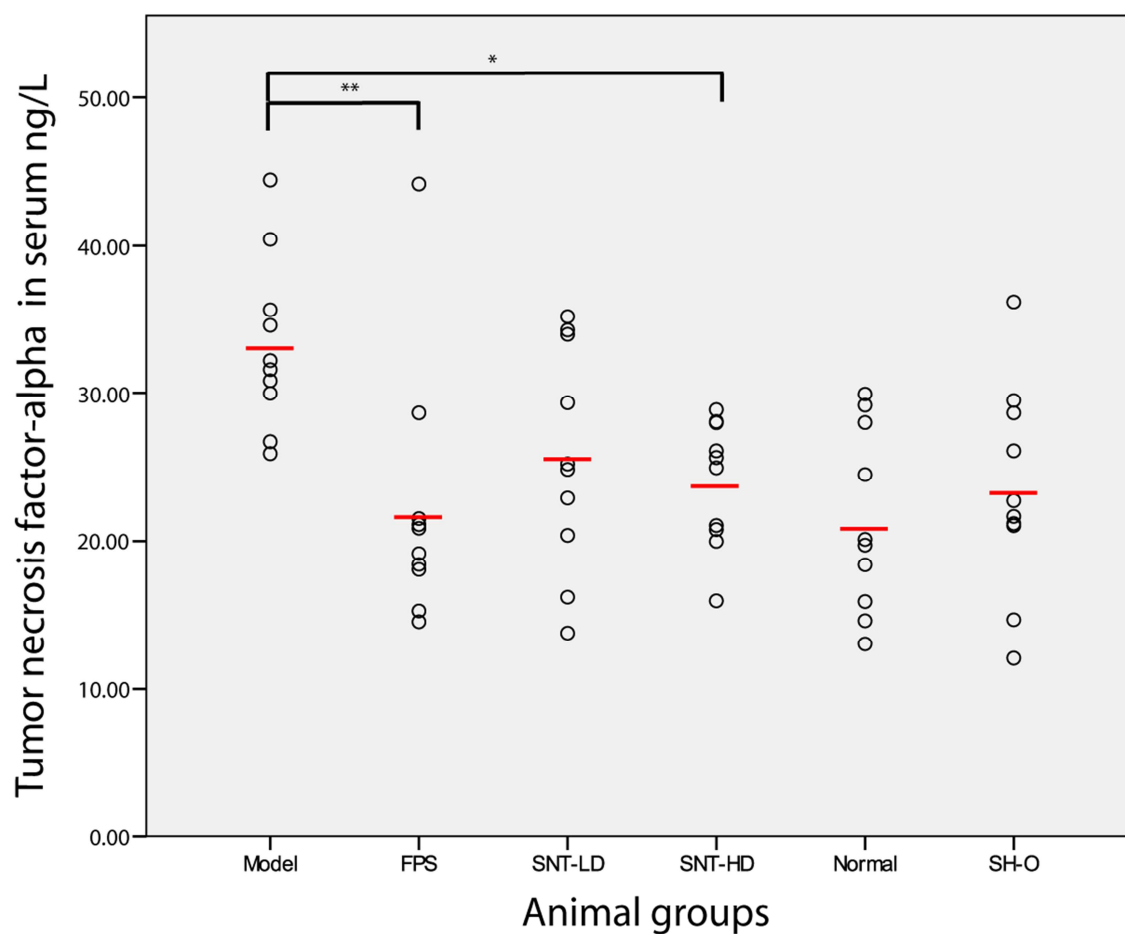


Figure 51. TNF- α in serum ng/L; * $P < 0.05$, ** $P < 0.01$; $n = 10$

Table 50. *TNF- α* in serum ng/L

Animal groups	N	Minimum	Maximum	Mean	Std. Deviation	Variance
Model	10	25.92	44.40	33.23	5.78	33.38
FPS	10	14.52	44.14	22.17	8.66	74.91
SNT-LD	10	13.75	35.19	25.61	7.57	57.25
SNT-HD	10	15.94	28.86	23.94	4.27	18.25
Normal	10	13.01	29.92	21.33	6.21	38.54
SH-O	10	12.06	36.18	23.38	7.10	50.47

n = 10

Table 51. Evaluation of the significant value from multiple group comparison of *TNF- α* level in serum

Comparison of groups	Significance
Model vs. FPS	0.007
Model vs. SNT-LD	0.135
Model vs. SNT-HD	0.036
Model vs. normal	0.003
Model vs. SH-O	0.022
FPS vs. SNT-LD	0.862
FPS vs. SNT-HD	0.992
FPS vs. normal	1.000
FPS vs. SH-O	0.999
SNT-LD vs. SNT-HD	0.994
SNT-LD vs. normal	0.716
SNT-LD vs. SH-O	0.976
SNT-HD vs. normal	0.953
SNT-HD vs. SH-O	1.000
Normal vs. SH-O	0.984

Numbers in bold = $P < 0.05$

High sensitivity C-reactive protein in serum

The mean values of hs-CRP in the serum (mg/L) of all groups are shown in figure 52 and table 52. There was a significant difference for hs-CRP in the following groups (table 53): model x FPS and SNT-LD and SNT-HD (6.71 ± 0.70 mg/L vs. 5.02 ± 0.71 , 3.95 ± 0.61 , 3.97 ± 0.77 mg/L, respectively); FPS x SNT-LD and SNT-HD which showed lower levels, even lower than the normal and SH-O groups (4.80 ± 0.52 mg/L and 4.53 ± 0.66 mg/L).

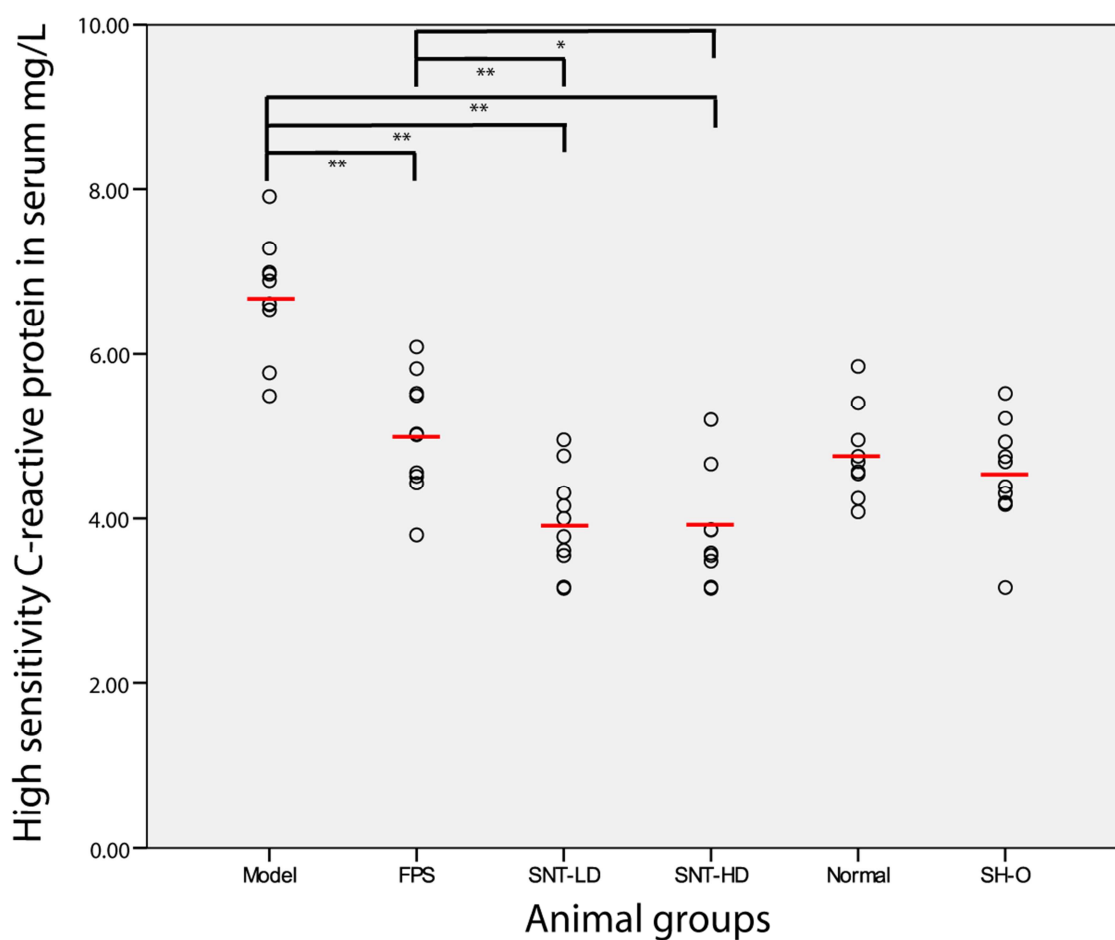


Figure 52. Hs-CRP in serum mg/L; * $P < 0.05$, ** $P < 0.01$; $n = 10$

Table 52. Hs-CRP in serum mg/L

Animal groups	N	Minimum	Maximum	Mean	Std. Deviation	Variance
Model	10	5.49	7.91	6.71	0.70	0.49
FPS	10	3.80	6.08	5.02	0.71	0.51
SNT-LD	10	3.15	4.95	3.94	0.61	0.38
SNT-HD	10	3.15	5.20	3.97	0.77	0.60
Normal	10	4.08	5.85	4.80	0.52	0.28
SH-O	10	3.16	5.52	4.53	0.66	0.43

n = 10

Table 53. Evaluation of the significant value from multiple group comparison of hs-CRP in serum

Comparison of groups	Significance
Model vs. FPS	0.000
Model vs. SNT-LD	0.000
Model vs. SNT-HD	0.000
Model vs. normal	0.000
Model vs. SH-O	0.000
FPS vs. SNT-LD	0.008
FPS vs. SNT-HD	0.011
FPS vs. normal	0.975
FPS vs. SH-O	0.565
SNT-LD vs. SNT-HD	1.000
SNT-LD vs. normal	0.061
SNT-LD vs. SH-O	0.376
SNT-HD vs. normal	0.077
SNT-HD vs. SH-O	0.434
Normal vs. SH-O	0.942

Numbers in bold = $P < 0.05$

5.2.3.4 Effects of SNT on vascular active markers: endothelin-1 and nitric oxide

Endothelin-1 (ET-1) is a potent vasoconstrictor which is synthesized in the vasculature and myocardium by various cell types and which is associated with the development of cardiac dysfunction. It stimulates myocyte hypertrophy *in vitro* and greatly influences the synthesis and degradation of the extracellular matrix ^{284,300}. In general, ET-1 is regarded as a biomarker released only in connection with the observation of hypertrophic response or other hypertrophic stress ³⁰¹. ET-1 at high plasma concentrations predicts an outcome of chronic heart failure ^{284,300}. Nitric oxide (NO) acts as a key contributor to vascular health due to many effects such as limiting vascular inflammation, platelet aggregation, monocyte adhesion to endothelial cells and abnormal smooth muscle cell proliferation ³⁰²⁻³⁰⁴. Once diffused to vascular smooth cells, it activates guanylate cyclase which results in vasodilation ³⁰⁵. Endothelial dysfunction is connected to a decreased bioavailability of vasodilators, especially NO ³⁰⁴.

Endothelin-1 in serum

Endothelin-1 levels in serum (ng/L) are presented in figure 53. No statistical differences between the groups were observed (table 55). The mean value of SNT-HD (158.49 ± 9.97 ng/L) was lower in comparison to all other groups and close to that of the normal group (161.40 ± 13.95 ng/L) (table 54).

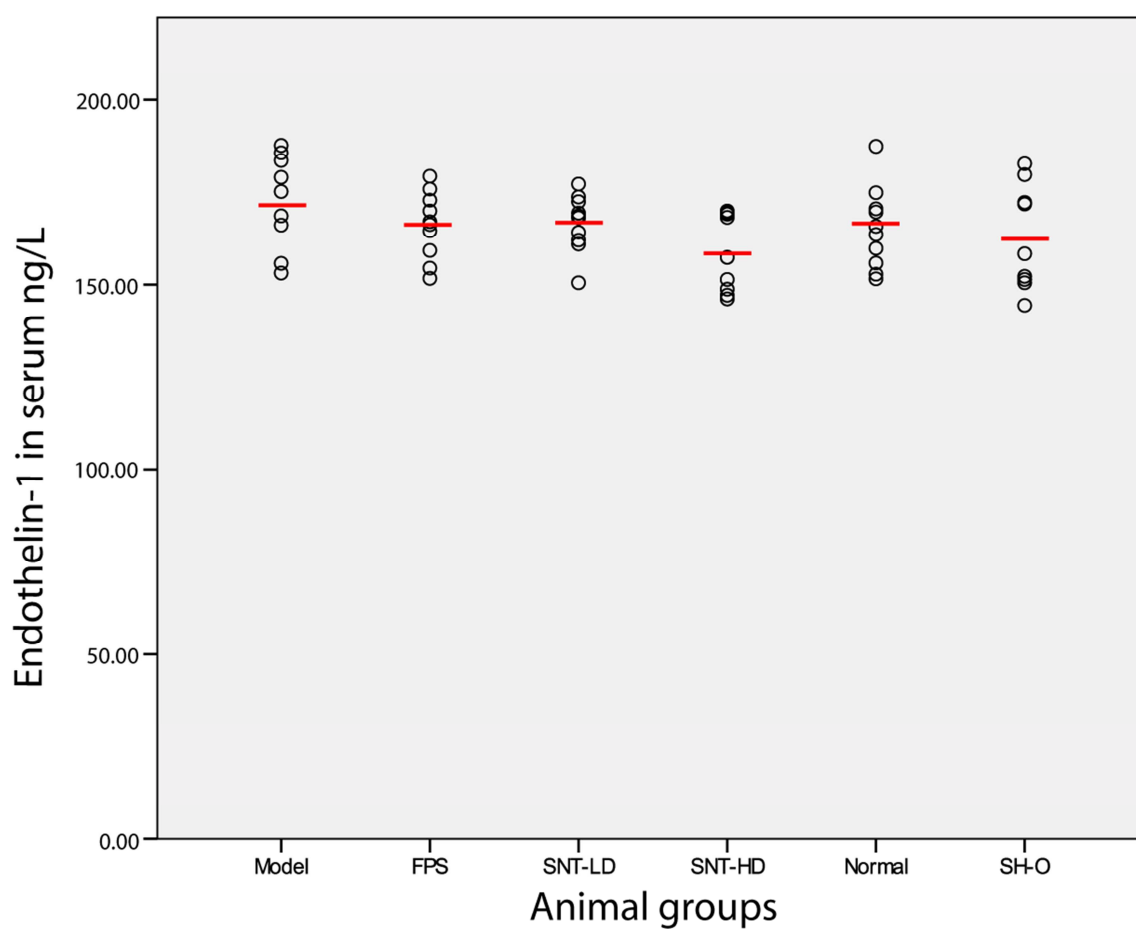


Figure 53. ET-1 in serum ng/L; $n = 10$

Table 54. *ET-1* in serum ng/L

Animal groups	N	Minimum	Maximum	Mean	Std. Deviation	Variance
Model	10	153.10	187.77	171.09	13.17	173.43
FPS	10	151.65	179.41	166.15	9.01	81.13
SNT-LD	10	150.48	177.27	166.74	7.70	59.25
SNT-HD	10	146.11	169.99	158.49	9.97	99.49
Normal	10	151.54	187.51	165.20	11.07	122.53
SH-O	10	144.36	182.80	161.40	13.95	194.52

n = 10

Table 55. Evaluation of the significant value from multiple group comparison of *ET-1* in serum

Comparison of groups	Significance
Model vs. FPS	0.915
Model vs. SNT-LD	0.949
Model vs. SNT-HD	0.127
Model vs. normal	0.838
Model vs. SH-O	0.376
FPS vs. SNT-LD	1.000
FPS vs. SNT-HD	0.633
FPS vs. normal	1.000
FPS vs. SH-O	0.928
SNT-LD vs. SNT-HD	0.556
SNT-LD vs. normal	1.000
SNT-LD vs. SH-O	0.886
SNT-HD vs. normal	0.750
SNT-HD vs. SH-O	0.991
Normal vs. SH-O	0.971

Nitric oxide in serum

The mean values of NO in serum ($\mu\text{mol/L}$) of all groups are shown in figure 54 and table 56. A remarkably higher mean value of the SNT-HD group could be seen compared to all other groups with a significant difference to the model group ($64.85 \pm 8.74 \mu\text{mol/L}$ vs. $48.48 \pm 1.01 \mu\text{mol/L}$). No statistical difference could be seen in the other groups (table 57).

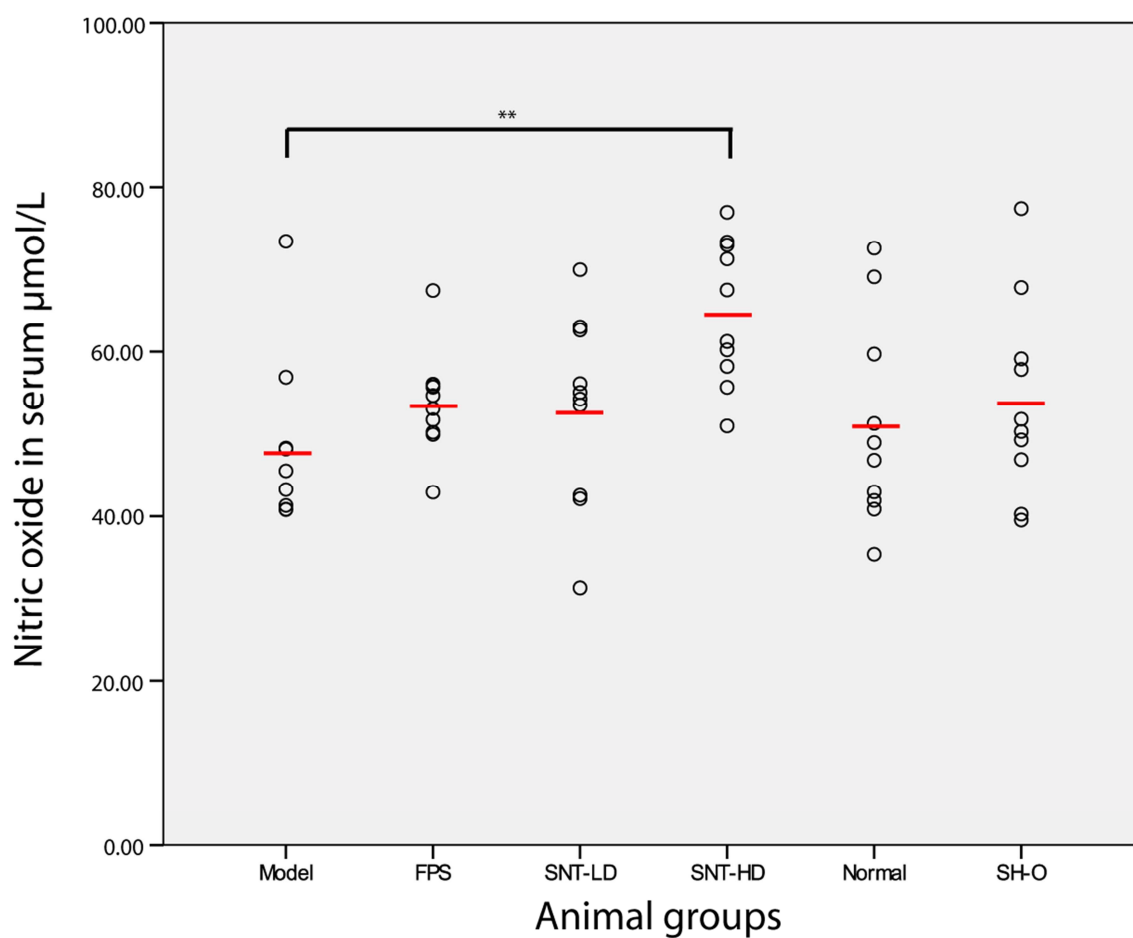


Figure 54. NO in serum $\mu\text{mol/L}$; ** $P < 0.01$; $n = 10$

Table 56. NO in serum $\mu\text{mol/L}$

Animal groups	N	Minimum	Maximum	Mean	Std. Deviation	Variance
Model	10	40.80	73.43	48.18	1.01	102.83
FPS	10	42.92	67.43	53.78	6.24	38.91
SNT-LD	10	31.24	69.97	53.06	11.54	133.13
SNT-HD	10	51.01	76.98	64.85	8.74	76.39
Normal	10	35.38	72.59	50.96	12.41	153.98
SH-O	10	39.53	77.42	54.04	11.88	141.02

n = 10

Table 57. Evaluation of the significant value from multiple group comparison of NO in serum

Comparison of groups	Significance
Model vs. FPS	0.832
Model vs. SNT-LD	0.898
Model vs. SNT-HD	0.009
Model vs. normal	0.991
Model vs. SH-O	0.804
FPS vs. SNT-LD	1.000
FPS vs. SNT-HD	0.180
FPS vs. normal	0.990
FPS vs. SH-O	1.000
SNT-LD vs. SNT-HD	0.130
SNT-LD vs. normal	0.998
SNT-LD vs. SH-O	1.000
SNT-HD vs. normal	0.045
SNT-HD vs. SH-O	0.200
Normal vs. SH-O	0.985

Numbers in bold = $P < 0.05$

5.2.4 Pathological description of the hearts

For the pathological description, the hearts were removed from the chest. The heart samples were fixed in 10 % neutral buffered formaldehyde solution and embedded before being cut into 4 μm section and stained with hematoxylin and eosin. Statistical evaluation for the pathohistological description of the hearts and livers was not possible because the original paraffin blocks are not yet available, therefore only histological images could be evaluated.

The morphologic changes of each group are presented in figure 55. A difference in the wall thickness of the ventricles between the model group and all other groups is quite obvious. The rats of the model group showed evidence of an increase in cardiac dilatation associated with the follow up of MI, whereas SNT had prevented the development of chamber dilatation post-MI. This observation backs up the results obtained with echocardiography where LVDd and LVSD of the FPS, SNT-LD and SNT-HD groups were lower and the calculated EF higher, partly significantly, than those of the model group.

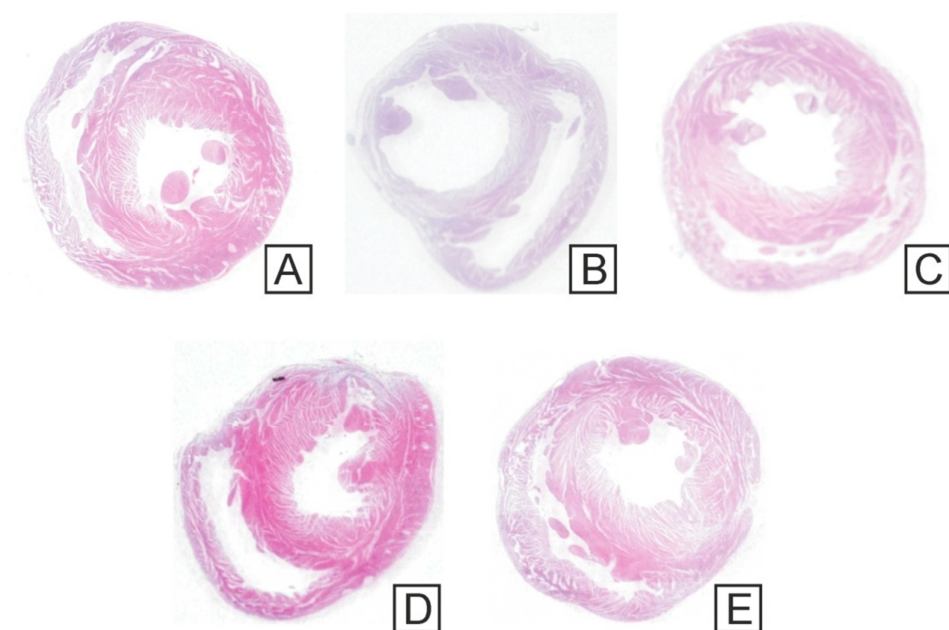


Figure 55. Effects of the treatment with SNT on the cardiac structure in rats

- A = SH-O = sham-operated group (sham-operation, treatment: water)
- B = Model = model group (induction of AMI, treatment: water)
- C = FPS = fosinopril group (induction of AMI, treatment: 900 $\mu\text{g/kg}$ fosinopril)
- D = SNT-LD = SNT-low dosage group (induction of AMI, treatment: 4.5 g/kg SNT)
- E = SNT-HD = SNT-high dosage group (induction of AMI, treatment: 13.5 g/kg SNT)

5.2.5 Histological description of heart and liver

5.2.5.1 Heart

On the basis of the photomicrographs in figure 56 no conspicuous differences could be seen in the histology of the hearts. The original paraffin blocks are not yet available, therefore only these photomicrographs could be used for the analysis.

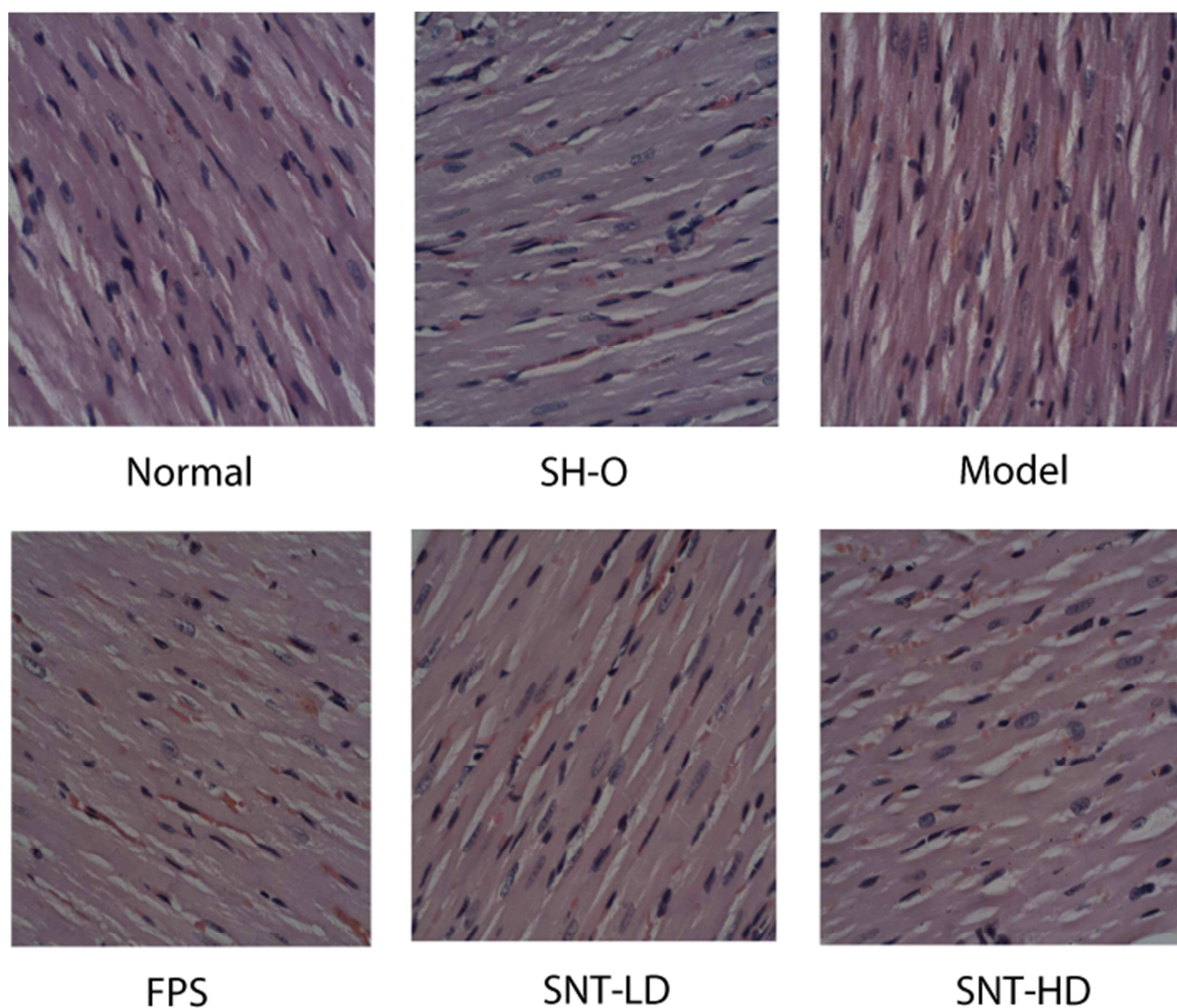


Figure 56. Photomicrographs of the heart ventricles; all photomicrographs are enlarged 40x15 times.

Normal = blank group (no operation, no treatment)

SH-O = sham-operated group (sham-operation, treatment: water)

Model = model group (induction of AMI, treatment: water)

FPS = fosinopril group (induction of AMI, treatment: 900 μ g/kg fosinopril)

SNT-LD = SNT-low dosage group (induction of AMI, treatment: 4.5 g/kg SNT)

SNT-HD = SNT-high dosage group (induction of AMI, treatment: 13.5 g/kg SNT)

5.2.5.2 Liver

No lobar structural differences could be observed between the groups (figure 57). Liver cells were normal in all groups and there was no sign of infiltration of inflammatory cells. Furthermore it can clearly be seen that there was no fibrosis and no accumulation of bile pigments. These results indicate that the treatment of low as well as high dosage of SNT over a period of 30 days did not affect the liver cells. In addition, it backs up the results obtained by HPLC analysis in which no diester-diterpenoid alkaloids could be detected in the SNT decoction administered to the rats.

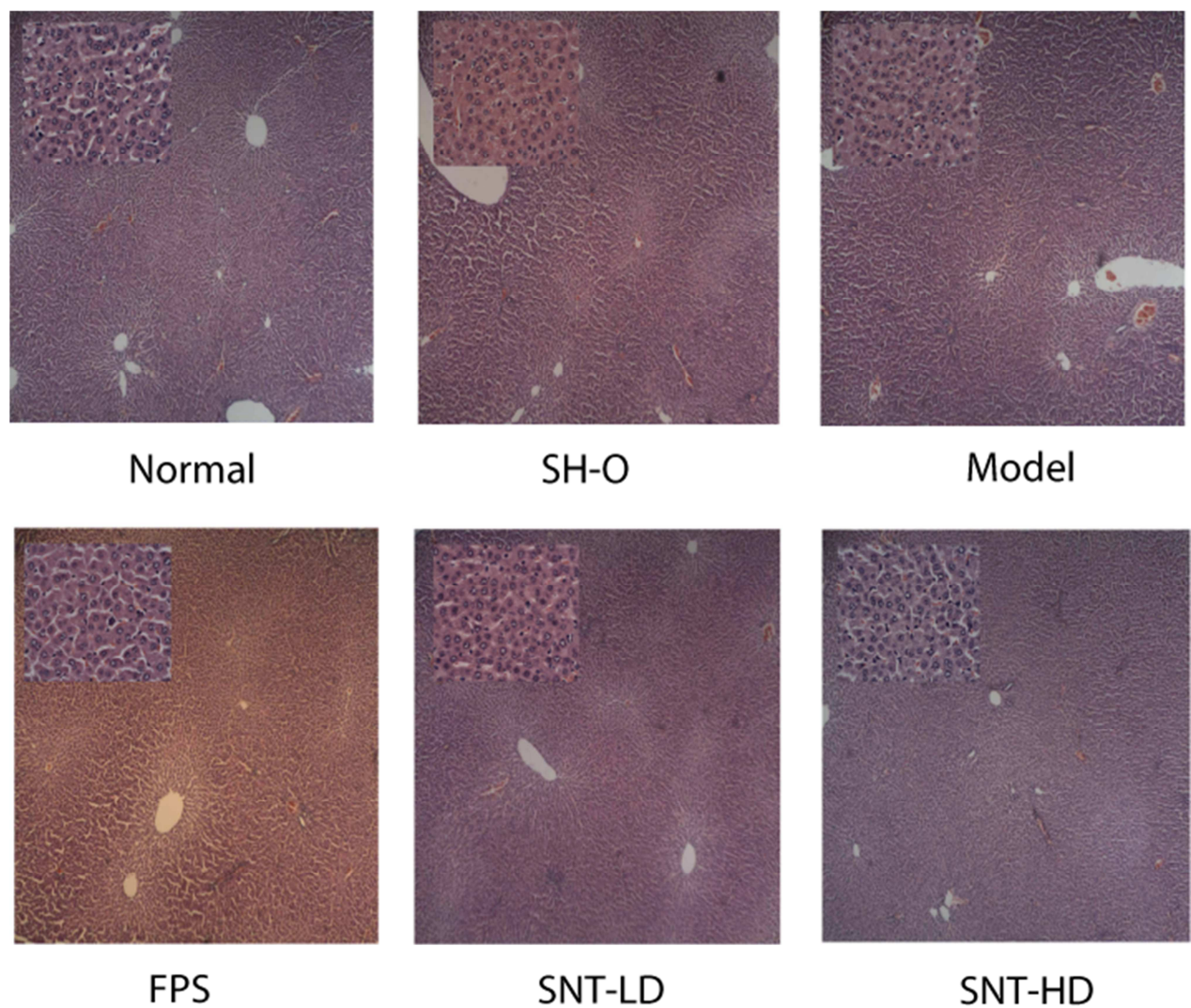


Figure 57. Hematoxylin and eosin stained liver sections; all photomicrographs are enlarged 4x15 and 40x15 times (small detail of each group).

Normal = blank group (no operation, no treatment)

SH-O = sham-operated group (sham-operation, treatment: water)

Model = model group (induction of AMI, treatment: water)

FPS = fosinopril group (induction of AMI, treatment: 900 μ g/kg fosinopril)

SNT-LD = SNT-low dosage group (induction of AMI, treatment: 4.5 g/kg SNT)

SNT-HD = SNT-high dosage group (induction of AMI, treatment: 13.5 g/kg SNT)

6. Discussion

6.1 Quality control and analysis of the effective components

Heavy metal constituents

Twenty-four batches of the medicinal herbs pretreated *A. carmichaelii*, *Z. officinale*, *C. cassia* as well as untreated and pretreated *G. uralensis* were tested for the heavy metal analysis. The results obtained indicate that the concentrations of lead (Pb), cadmium (Cd) and mercury (Hg) do not exceed the limits of 5.00 mg/kg Pb, 1.00 mg/kg Cd and 0.1 mg/kg Hg recommended for medicinal plants by the European Pharmacopoeia²⁵⁸ in any of the batches. Besides, the case example used for the calculations shows that the consumption of each combination of the tested herbal batches for SNT would show arsenic (As), Pb, Cd and Hg concentrations lower than the total sum of the provisional tolerable weekly intake (PTWI) established by the Joint Food and Agriculture Organization/World Health Organization Expert Committee on Food Additives²⁵⁹. However, As and Pb would account for around 30 % of the PTWI and an additional intake through food and drink should be taken into account.

In the samples used for the decoction of *in vivo* experiments (batch number 5 of each herb) it was apparent that nearly all concentrations of detected elements were lower, sometimes considerably lower than in the batches from Shandong Province. The difference of the ion concentrations observed in batches with different plant origin indicates an influence of the place of origin on the concentration of the ions.

The element concentrations of the four batches (batch number 1 – 4) from Shandong Province sometimes differed remarkably within one herbal group. This might be due to the different exposure of parts of plant i.e. an unequal load of heavy metal composition in air or dust compared to that of the soil and therefore a difference in the accumulation of the elements in the bark, rhizome or roots.

Summarized, the concentrations of As, Pb, Cd and Hg determined in the herbal material of SNT were lower than the permissible levels. In respect to this, we must be aware that the analysis can only present the status quo of the elements but is not in relation to the original presence of the elements in the herbs. Various studies have reported that the concentrations of As, Cd and Pb

observed in herbal infusion are low due to the solubility in water compared to those of raw herbal material ^{28,30,306}. Nevertheless, from the concentrations determined in the raw herbal material digested with concentrated nitric acid in a microwave reaction system, neither can conclusions be drawn from the presence of the elements in the decoction of SNT nor from the amount of elements which are absorbed by the human body.

Natural products in SNT

For the qualitative fingerprinting analysis of the Si Ni Tang decoction (SNT), a method with successful peak-separation was established. Only one other study on the chemical constituents of SNT has been documented to date. In that experiment, six compounds were isolated and identified as vanillic acid, formononetin, isoliquiritigenin, liquiritigenin, kumatakenin B and uracil ³⁰⁷. In our study also liquiritigenin was detected and in addition liquiritin, isoliquiritin, glycyrrhizic acid and eugenol were identified. Liquiritin and glycyrrhizic acid were found to be the dominant components of the standards analyzed in this SNT decoction.

Detection of aconitines in *Aconitum carmichaelii*

SNT consists of parts of the four plants *Glycyrrhiza uralensis*, pretreated *Aconitum carmichaelii*, *Zingiber officinale* and *Cinnamomum cassia*. However, *A. carmichaelii* is highly toxic due to diester diterpene alkaloids (DDAs) such as aconitine (AC), hypaconitine (HA) and mesaconitine (MA). The HPLC-DAD method developed (figure 58) for the identification and quantification of AC, HA and MA from natural sources showed good repeatability, precision and recovery and thus provides a reliable basis for the quantification of the DDAs in extracts of *A. carmichaelii*. The quick analysis time of 23 min per passage should be particularly emphasized, as it is much faster than other published HPLC methods for the analysis of AC, HA and MA, e.g. 45 min ²⁴⁷, 75 min ²⁶⁸, 80 min ²⁶⁶ and 85 min ⁶⁹. The advantage of this new method could be used for the standardized quality control of *Aconitum* species, allowing a high throughput in a very short time.

In this project only HA could be detected in the batches we examined. This coincided with previous studies of *A. carmichaelii* roots, where HA showed the highest concentration of the

diester diterpene alkaloids (DDAs)^{69,266,267,308}. This observation leads to the assumption that HA is most abundant in the roots and tubers of *A. carmichaelii*.

The influence of extraction conditions on the DDAs

To recapitulate, the structural differences of the three aconitines, AC, HA and MA, lie in the substituents at C₃ and the N-atom (figure 3, section 3.1.3). The influence of the conditions of extraction, in particular the solvent and the pH, on the efficacy of the extraction of the aconitines has been investigated in previous studies^{231,247,266,309,310}. The most efficient extraction solvent in our study was methanol (269.34 µg/g HA), whereas using 1 % hydrochloric acid only 93 % of HA (251.12 µg/g HA) were detectable. As a matter of fact, Csupor et al. reported that in their experiments 1 % HCl was by far the most effective extraction solvent followed by the neutral solvent MeOH-H₂O (9:1), where only 50 % of the aconitines could be determined²⁴⁷. Similar results were obtained by Koop in 2007 when 80.1 µg/g HA, 36.0 µg/g MA and 5.3 µg/g AC (mean, n=6) were determined in Fu Zi batches extracted using aqueous HCl^{75,308}.

Previous studies indicate that the extraction and elution conditions using alkaline solvents are not recommended due to the observation of a fast hydrolysis in alkaline solution^{309,310}, especially of AC and MA²³¹. This was the reason for the decision to conduct the experiments for this thesis using both neutral and acidic solvents. In acidic buffer solutions an additional decomposition of AC and MA around 50 % occurred, whereas HA stayed stable²³¹. This might explain the fact that there were not any AC and MA detectable when using pH 3 in these experiments. As these studies show, there was always an issue of degradation of the aconitines whether alkaline or acidic, aqueous or organic solvents were used. It must also be mentioned that this set of studies were difficult to compare and it was a challenge to determine the best extraction condition. Nevertheless, the method established (figure 58) is distinctive for its very short analysis time, therefore significantly minimizing the time and costs for quality control.

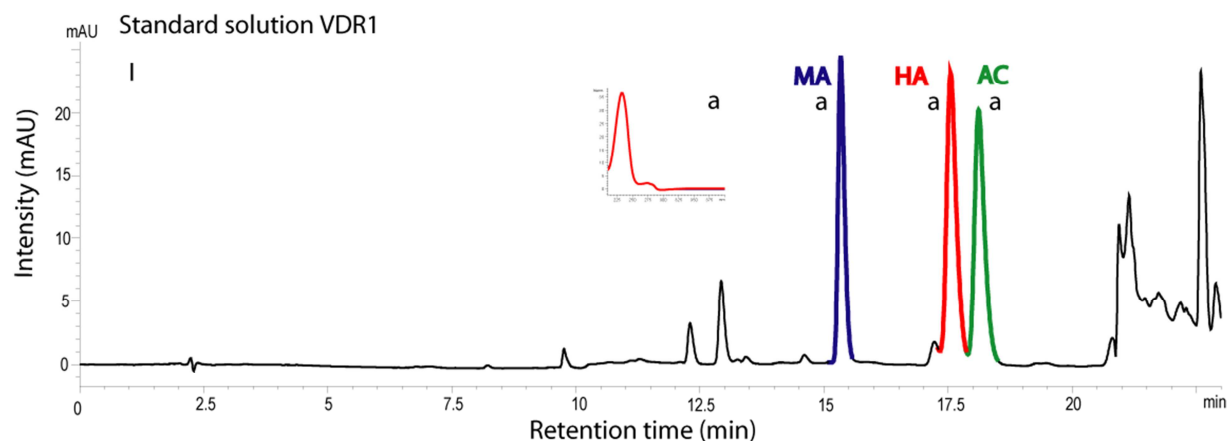


Figure 58. HPLC profile of the established method, standard solution VDR1, a = UV-spectra of aconitine, hypaconitine and mesaconitine, monitoring wavelength 233 nm

Stipulation and risk of AC in the Chinese Pharmacopoeia

In the Chinese Pharmacopoeia, 2010, an upper limit of only 0.02 % of AC is stipulated⁴⁶. In fact, AC was often lower than 0.02 % or undetected in processed *Aconitum* batches²⁶⁸, due to its non-appearance in the batch analyzed, a less general stability of AC compared with HA and MA and/or inefficient extraction conditions. In fact, HA and MA concentrations were found in these samples, but not considered as a safety risk in application of the medicine according to the Chinese Pharmacopoeia. However, it is well-known that HA and MA are as toxic as AC^{75,311} and Qin et al. also questioned this attitude when he determined a greater toxicity in the *Aconitum* samples than the detected contents of AC, MA and HA would implicate³¹². Therefore, even the concentration of the three DDAs could not be responsible for the total toxicity found in the *Aconitum* species as it is commonly used³¹².

The influence of pretreatment on the DDAs

Various studies were performed on the ability to pretreat *Aconitum* samples to reduce the DDAs and to transform them into less toxic or non-toxic derivatives^{69,310,313}. *Heishunpian*, the most wide-spread and favored Fu Zi processing method, showed the lowest concentration of AC, MA and HA in comparison to eight other pretreated Fu Zi samples (using different processing

methods)⁶⁹. As described earlier in section 3.2.1, it has been reported that during the processing the ester bindings of the DDAs at C₈ and C₁₄ are hydrolyzed and as a result the toxicity is decreased^{73,232,233}. This might be confirmed by these results, in which HA was detected in only two out of eight processed *A. carmichaelii* batches and the amounts in these two samples being three to four times lower than those detected in the unprocessed batch. Similar results were reported by Csupor et al. when AC, HA and MA were detected in unprocessed *A. carmichaelii* using LC-MS, whereas no aconitine-type alkaloids were determined in the processed *A. carmichaelii* samples²⁴⁷.

Various studies have been published concentrating on the establishment of a precise and quick method to identify and quantify the aconitines, AC, HA and MA, of the *Aconitum* species. Furthermore, diverse pH values and several extraction solvents have been tested and compared to improve the established method by the implementation of more effective extraction conditions. It can be concluded that there is, of course, a non-negligible influence of the solvent and pH on the extracted concentration of AC, HA and MA. So far there has been no standardized method established yet for the quantification of the three highly toxic aconitines, although it is highly essential.

In this respect we should pay attention to the method developed in this project for the quantification of AC, HA and MA. This method not only provides a reliable basis for the quantification of the DDAs, but its particular, distinctive feature is its quick analysis time, allowing a high throughput of *Aconitum* extracts in a relatively short time. There is also the consideration that the extraction with organic eluents might even be unnecessary for aconite roots used for the decoction. Firstly, the extraction may lead to a false negative result due to decomposition of the DDAs as reported by several investigators mentioned above. Secondly, when water-extracted (decoction), the analysis is much more practice-orientated: the water-soluble fraction will be detected which is closer to the decoction which is given to humans. In the case of decomposition through water extraction, this would also have already been respected. In other words, the amount of the DDAs effectively detected is the dose suitable to human consumption and therefore it is a straightforward method to determine if the medicinal herb is safe to use.

Is there a clinical effect without the DDAs?

Another question of interest is: Is there a clinical effect without the DDAs? Various studies call attention to the necessity of a specific concentration of DDAs that should not be completely alleviated in order to achieve the desired clinical effects^{75,314,315}. Little is known about the pharmacological effects and the toxicity of the great amount of DDA-degradation products. A significant anti-inflammatory effect and analgesic action is reported of benzoylmesaconine (the hydrolyzed correspondent of MA) by Suzuki et al.³¹⁶. Ameri A. concluded in her study on the structure-dependent inhibitory action of 14-benzoyltalitasamine and talitasamine in rat hippocampal slices, that the existence of a benzoylester group is essential to show pharmacological effects, whereas alterations at C-3, C-6, C-8 and C-13 cause different effects³¹⁵. Ten diterpenoid alkaloids were isolated from *A. carmichaelii* and their cytotoxic activities tested on several human cancer cell lines by Gao et al.³¹⁷. The results indicate that the presence and the quantity of ester groups play a decisive role in the anticancer potency of these DDAs³¹⁷. Besides, the claim that aconite has a positive influence on heart related diseases when administered in the optimal dose, is presumably not only due to the aconitine-like alkaloids but also to isoquinoline derivative higenamine which shows inotropic effects^{75,318}.

Further investigations are to be recommended on the mode of interaction and the mechanism of detoxification. Screening of the toxicity of the degradation products and the analysis of their pharmacokinetics and –dynamics is also imperative to guarantee safe and efficient medicinal use.

Interaction between aconitine and other natural products

In TCM it is common to treat patients individually. A precise differentiation of both, the patient's pattern of symptoms and the nature and properties of individual herbs, are utilized for effective treatment. It is therefore usual to prescribe unique combinations of formulas that comprise selected herbs with specific characteristics and functions that act on the particular symptoms diagnosed³⁷. Furthermore, it has been experienced that a proper combination with other herbs results in a reduction of possible side effects and/or toxic impacts³⁷.

Some studies have documented interactions between herbs combined in a formulation^{36,319-322}. Sui et al. reported that the *Aconitum* alkaloids of Fu Zi decreased when co-decocted with other

herbs, whereas Wang et al. reported both a reduction and elevation of DDAs when *Aconitum* was combined with other herbs³²⁰. Qin et al. studied if a co-administration of *Paeonia lactiflora* could significantly reduce the acute toxicity of *A. carmichaelii*³²³ and found that the combination showed an even better effect on the treatment of arthritis in rats^{36,324}. Various studies deal with the influence of multicomponent on the bioavailability and absorption of the DDAs^{321,322,325} which might be due to the ability of various constituents to alter the pharmacokinetics of Fu Zi, and in particular the DDAs, but very little is known about the specific mode of the interaction.

While most investigators have concentrated on the reduction of DDAs through degradation, in this project a strong focus was placed on the analysis of the reduction through complexation. As mentioned in section 3.2.2, only a few research groups have investigated the ability of licorice and ginger to interact with the aconitines. In general, experiments concentrating on the interaction were carried out in China, and only abstract information was available in English. The available information shows that the investigators agreed on the ability of *Radix Glycyrrhizae* to reduce the content of the aconitines²³⁷⁻²⁴⁴. Huang et al. alone reported on the potency of *Rhizoma Zingiberis* to decrease the aconitines²³⁷. So far, nothing has been published about the special mode of that particular interaction in English-language monographs, but our results concurred with the reports of other investigators. These observations clearly indicate that licorice may play a major role in reducing the DDAs in the SNT formula. Cinnamon and ginger may also contribute to the reduction of the DDAs.

Aconitine/liquiritin complex

Liquiritin, together with glycyrrhizic acid, is one of the major components of SNT analyzed in this project but isoliquiritin and liquiritigenin have also been detected in the SNT (see figure 14, section 5.1.2). According to our findings, liquiritin, isoliquiritin and liquiritigenin are primarily responsible for the interaction with the 62.10 µg HA/100 mg lyophilisate, detected in the single decoction of unprocessed *A. carmichaelii* (see figure 15 and table 16, section 5.1.4), but undetected in the SNT decoction containing the same concentration of the same batch of unprocessed *A. carmichaelii*. As a matter of fact, glycyrrhizic acid showed no effect on the concentration of the DDAs which is contrary to observations made in previous studies²⁴⁰⁻²⁴².

However, our results were consistent with the findings of some Chinese investigators. In his research, published in 2009, Shen et al. investigated the possibility that liquiritin might be one of the components responsible for the alleviation of the toxicity of aconite²⁴³. Zhang and Yang reported similar results on the ability of liquiritin to reduce the toxicity of *A. carmichaelii*²⁴⁴. It is assumed that bioactive compounds other than tested, of ginger (8-gingerol, 6-shogaol) and cinnamon (eugenol, cinnamaldehyde, 4-methoxy-cinnamaldehyde), are responsible for their ability to reduce the DDAs (see figure 17, section 5.1.5.1).

In conclusion, the stoichiometric ratio and binding constants for the complexation between liquiritin and aconitine in D₂O/MeOD (2:1) and (1:2), were estimated for the first time using titration experiments with ¹H-NMR. Due to the poor solubility of aconitine and liquiritin in water, it was not possible to perform these experiments using pure water as in decoction. By means of solubility tests it was found that 1/3 of MeOD in D₂O was necessary to dissolve the concentration of aconitine used and 2/3 of MeOD in D₂O was used as the concentration of liquiritin was increased for the analysis of the binding constants. The stoichiometry of 1:1 for the aconitine/liquiritin complex means that one molecule of aconitine interacts with one molecule of liquiritin. A balancing act was necessary to both, guarantee the accuracy of chemical shift detection and at the same time avoids the self-aggregation of aconitine. Due to the almost identical calculation of the binding constants, whether liquiritin/acnitrine in D₂O/MeOD (2:1) or aconitine/liquiritin in D₂O/MeOD (1:2), it was insignificant that we were obliged to only use the first titration points for the calculation of the binding constants of aconitine/liquiritin complexation because of the self-aggregation of aconitine at higher concentrations.

Binding constants are an indicator and used predominantly between less soluble drug substances and the carrier substance which has the function of introducing the medicinal drug to the site of its pharmacological action. The binding constants of the aconitine/liquiritin complex were calculated to be 4000 ± 600 L/mol and 3000 ± 300 L/mol. These values are in the range of the calculated binding constants of a complexation between econazole (an antifungal agent) and cyclodextrin which is commonly used as carrier substance³²⁶. The errors of 10–15 % observed are usual in these NMR titration experiments and may be higher in the case of liquiritin/acnitrine, seemingly caused by measurement errors from small changes in the proton chemical shifts of aconitine's H-12 at 0.2 mM (see figure 28, table 23, section 5.1.6.2).

The results obtained by UV-VIS measurements indicate that the molecular regions were the chromophores of aconitine and liquiritin are placed may not actually be strongly involved in the binding of the complex formed or that the binding behavior between aconitine and liquiritin was too small to get further information through analysis with UV detection.

At this point, there was evidence that a strong interaction occurs between aconitine and liquiritin with relatively high binding constants. The results obtained by 2D ROESY experiment did not show distinct dipolar couplings between the two molecules, aconitine and liquiritin, and this behavior was probably caused by a relative short binding time, insufficient for an intermolecular magnetization transfer. It is not possible to draw any conclusions at present about the substituents of the molecules involved in the interaction and nothing can be reported on the structure of the complex.

As a point of interest, liquiritin, liquiritigenin and isoliquiritigenin were isolated from processed tubers of *A. carmichaelii* for the first time in 2005 by Shim et al.³²⁷. Liquiritin and liquiritigenin were again isolated from processed aconite tubers by Lyu et al. in 2008³²⁸. This evidence may explain the very high binding constants found in our experiment. There is also the possibility that complexation can already occur during extraction and thus reduce the concentration of aconitine. It would be interesting to find out more about the biological function of liquiritin in the aconite tubers.

Our findings open new fields in detoxifying the DDAs in the *Aconitum* species. This unique approach offers a new opportunity to facilitate the handling of these highly toxic components and it offers additional security. Up until now the investigations all concentrated on the degradation of the DDAs, nothing has so far been published about the mechanism of complexation of the DDAs.

Conclusion and Outlook

The complexation between aconitine and liquiritin provides an additional technique to reduce the DDAs. In the case of an incomplete reduction of DDAs, e.g. an improper pretreatment, the combination of aconite with licorice and/or liquiritin can offer extra reliability. This newly developed approach contains a lot of future potential. Liu et al. sums it up with his statement:

“*Aconitum* is considered as a ‘double-edged sword’ in TCM because it has both therapeutic and poisonous characteristics”³²⁹. To quote the words of Paracelsus: “*The dose makes the poison*” because there is a narrow safety margin between a therapeutic and a toxic dose of *A. carmichaelii*. As previously mentioned, the question was if there actually is a pharmacological effect without the presence of DDAs. Now there could be a chance that aconitine could be released gradually from the complex and that a positive therapeutic effect occurs without any toxic side effect because of this. By means of a patch clamp technique, the effect of the complex on the Na⁺ channels should be investigated to enable conclusions to be drawn on the toxicity of the complex formed with aconitine and liquiritin. Further studies are necessary to discover the pharmacological effect of the complex itself and its stability in an acidic environment especially as it is in the stomach. In addition, it would be interesting to observe if aconitine is released out of the complex in the body and, if so, the mechanism behind it.

6.2 In vivo experiments

In the present study we investigated the effects of Si Ni Tang (SNT) on post-infarction ventricular remodeling in rats. This Chinese herbal formula consisting, of the radix of *Aconitum carmichaelii*, the rhizoma of *Zingiber officinale*, the cortex of *Cinnamomum cassia* and the radix of *Glycyrrhiza uralensis*, has been examined in comparison to other treatments. Myocardial infarction (MI) was induced in rats by left anterior descending artery ligation. After 30 days of treatment of the six groups - model, fasinopril (FPS), SNT low dosage (SNT-LD), SNT high dosage (SNT-HD), sham-operated (SH-O) and normal groups - echocardiography was performed prior to their death. The heart and liver tissues were removed for pathohistological examinations and blood samples were taken for the analysis of the serum levels of various markers known to be involved in the process of cardiovascular remodeling.

Myocardial infarction and the pathological consequences

Heart failure as a result of MI encompasses a wide and complex spectrum of clinical and morphological characteristics³³⁰. The progressive physiological and anatomical transformation of the ventricle such as left ventricular (LV) dilation, eccentric hypertrophy and infarct scar

thinning correspond collectively to ventricular remodeling^{331,332}. In the first day post-infarction, a degradation of collagen fibers occurs and the myocytes undergo necrosis^{332,333}. Pro-inflammatory cytokines such as interleukin-6 (IL-6), tumor necrosis factor alpha (TNF- α) and interleukin-1 β (IL-1 β) are released by accumulated macrophages and monocytes in the necrosis tissue^{291,334}. The infarction begins to spread, collagen and collagen struts are degraded by necrosis leading to acute dilation and wall thinning, and finally to LV cavity dilation³³². Cardiac fibroblasts deposit collagen on the thinner tissue and a scar forms when sufficient collagen has accumulated^{332,333}. The non-infarcted LV continues to remodel, cardiomyocytes develop eccentric hypertrophy and this results in increased ventricular radius which leads to wall stress and, as a consequence an increased demand for oxygen demand³³⁵. At first, these compensations appear beneficial, but as the process continues a miss-match of LV volume and wall thickness occurs which leads to decreased LV ejection fraction and ultimately to heart failure³³².

Si Ni Tang and its protective effects on ventricular remodeling post-MI

SNT is a representative Chinese medicinal formula. It has a tradition for the treatment of cardiovascular disease which began between 150 and 219 A.D. and has been officially recorded in the 2010 edition of the Chinese pharmacopoeia⁴⁶. Various investigations were carried out; the results of which justifies the traditional use of SNT in the treatment of cardiovascular diseases (see section 3.1.2). These includes improvement of blood flow^{51,54}, improvement of electrocardiography^{53,59}, improvement of echocardiography^{48,336}, reduction of infarct areas³⁵, improvement of blood lipids⁵⁵, regeneration of the unbalanced myocardial energy metabolism³⁵ and improvement in the life quality of patients post-MI^{59,60}. It seems that the SNT acts simultaneously on several targets involved in the protection of the heart and vascular system. However, to our knowledge, no investigations have yet been done on the effect of SNT on ventricular remodeling post-MI by analyzing the selected biomarkers. The main interest of this study was to find out if and how SNT takes part in the complex mechanisms of ventricular remodeling by analyzing several biomarkers and carrying out echocardiographic investigation and pathohistological examinations.

Molecular biological investigations

Collagen markers: collagen I, collagen III and transforming growth factor- β 1

In the present study the mean value of collagen type I (col I) and collagen type III (col III) in serum in the SNT-LD and SNT-HD groups were significantly lower (65 % and 73 %, respectively) vs. the model group ($P < 0.05$). In the cardiac tissue, the mean values of col I and III were also reduced in the SNT-LD and significantly reduced in the SNT-HD vs. the model group ($P < 0.05$). The reduction observed in the tissue of the SNT-HD group was in the same range as in the serum which was 75 % of the col I level and 67 % of the col III level of the model group. Collagen synthesis is controlled by the transforming growth factor beta (TGF- β), which is secreted by macrophages and fibroblasts³³⁷ and which serves as a biomarker for ventricular remodeling and hypertrophy^{276,277}. In our study there were no differences observed between the mean values of TGF- β 1 in serum in the four groups that were compared. The mean value of TGF- β 1 in myocardial tissue was lower in the SNT-HD groups than in the model group but no significant difference was determined. These findings show that the treatment with SNT had a positive influence on the col type I and col type III regulation and therefore had a direct effect on the fibrosis formation. However, SNT had no effect on the TGF- β 1 level.

Inflammatory markers: interleukin-6, interleukin-1 β , tumor necrosis factor- α , high sensitivity C-reactive protein

This study shows reduced levels of IL-6, and significantly reduced levels of IL-1 β ($P < 0.05$), TNF- α ($P < 0.05$), hs-CRP ($P < 0.01$) in the serum of the groups treated with SNT as compared to that of the model group. Notably, the level of high sensitivity C-reactive protein (hs-CRP) was reduced in both the SNT-LD and the SNT-HD groups to nearly half of those in the model group and were even lower than the levels of the normal and SH-O groups. The IL-1 β level of myocardial tissue was also significantly reduced in the SNT-HD treated rats to 73 % of that determined in the model group ($P < 0.01$). These results clearly demonstrate an anti-inflammatory effect of SNT. It is also assumed that the effects of SNT may not be restricted to a reduction of the inflammatory damage but, as mentioned in section 5.2.3.3, IL-6, IL-1 β and TNF- α can be associated with a variety of activities such as the regulation of other biomarkers which are involved in the ventricular remodeling. Examples of this are an increase of MMP

activity, the regulation of integrins, an increase in natriuretic peptides²⁸⁹, an induction of myocyte intercellular adhesion molecule-1²⁹¹, and an activation of nitric oxide production^{269,292}.

Functional index markers: atrial natriuretic peptide and cardiac troponin T

In the present study, the atrial natriuretic peptide (ANP) levels determined in the serum were very similar in all the groups. The mean of cardiac troponin T (cTnT) in the serum of the groups treated with SNT was lower than that of the model group, although not significantly, and the examination of a larger number of subjects would be necessary to possibly discover significant differences between the model groups, and those treated with SNT. In this particular test no influence of SNT was found on these markers, which respond to wall stress.

Vascular active markers: endothelin-1, nitric oxide

No statistical differences in the ET-1 levels in the serum could be seen between the groups. As a matter of interest, Wu et al. observed in their study that SNT could significantly lower myocardial ET and they found by immunohistochemical examination, that this effect might be related to an inhibition of ET-1 gene expression⁵². A remarkably higher mean value of NO in the serum was observed in the SNT-HD group than in all the other groups, showing a significant difference to the model ($P < 0.01$) and the normal ($P < 0.05$) groups. This increase of NO is unanimous with a study of Liu et al. when a significantly increased content of NO in the culture medium of rat vascular endothelial cells treated with SNT could be seen³³⁸. Yang et al. reported on the regulating effect of SNT on blood pressure because it adjusts the expression levels of endothelin receptor-A and endothelial nitric oxide synthesis in the heart, and the vasoactive marker levels (ET and NO) in the blood³³⁹. To conclude our observations, ET-1 – a vasoconstrictor, characterized as a biomarker for hypertrophic response³⁰¹ – was not affected. However, NO, which is known to limit vascular inflammation, platelet aggregation, monocyte adhesion to endothelial cells and abnormal smooth muscle cell proliferation³⁰²⁻³⁰⁴, was affected by the treatment with SNT.

Effect of treatment with SNT on body weight and heart weight

A statistically significant difference between the body weights and heart/body weight ratio of each individual group was not noticed. The heart/body weight ratio did not appear to have an effect on the development of significant hypertrophy in the model group. However, a great difference could be observed between the female and the male rats: both gained weight during the experiment, but the female rats had in total higher heart/body weight ratios due to less body weight, though the heart weights were similar to those of the male rats. The pathological description of the hearts did not show a difference in fibrosis when comparing male and female rats, therefore this observation remains inexplicable.

Echocardiographic analysis and pathological description of the heart and liver

In the present study, it can be seen that the administration of SNT had a beneficial effect on ventricular remodeling as was indicated by the echocardiographic tests performed 31 days post-MI. The treatment with SNT decreased left ventricular dilation as indicated by reduced left ventricle end-diastolic dimensions and left ventricle end-systolic dimensions. In addition, the significantly improved ejection fraction in the groups treated with SNT resulted from an enhancing effect of the SNT on the left ventricular function. Tan et al.³⁵ and Dietl et al.³³⁶ documented the same echocardiographic outcome in their study. These findings were backed up by the increase of cardiac dilatation associated with MI at follow up in the model group, whereas SNT had prevented the development of chamber dilatation post-MI, as was observed in the pathological examination of the hearts. No conspicuous differences could be seen in the histology of the hearts when analyzing the available photomicrographs. The histological description of the rat livers showed that there were no pathological changes of the lobular structures of groups treated with SNT, indicating the safe use of SNT.

Effectiveness of the selected biomarkers analyzing the effects of SNT on cardiac remodeling

Based on the concepts of systems biology, this study confirms that most of the prognostic biomarkers used post-MI are very effective. However, the levels of the four markers, ANP, IL-6,

ET-1, TGF- β 1, were not found to be significantly different in the model group than in the normal and SH-O groups. The assumption was there were too few subjects involved to find a significant difference. In general, significant differences could not be detected in any of the determined biomarker levels between the normal and the SH-O groups, suggesting that the operation did not influence the marker expressions. To sum this up, the selected biomarkers enabled analysis of the effects of SNT on cardiac remodeling and correspond to the improving effect on the LV function and to the development of cardiac dilatation being prevented, as was observed in the echocardiography and pathological examinations. This study supports the cardiologic research by providing an effective method of screening to gain insight into the drug's influence on cardiac remodeling post-MI. The method of analysis has proved successful and therefore should aid the development of conventional pharmaceutical as well as the research on TCM.

Inflammatory cytokines and collagen I and III

Even though the effects of the pro-inflammatory cytokines were found to be controversial, either resulting in healing or resulting in acute cardiac rupture²⁸⁹, it can be summarized that the treatment with SNT showed beneficial effects. The reduced, partly remarkably reduced, collagen and inflammatory marker expressions in the groups treated with SNT deserve a special mention, since the levels of col I and III, IL-1 β , TNF- α and hs-CRP observed were almost the same as those of the normal groups, showing no statistical differences. Combining these facts with echocardiographic and pathohistological results, SNT shows effects comparable to those of the fasinopril (FPS) treated group.

Figure 59 demonstrates the induction of inflammatory cytokines after myocardial ischemia and their effect on acute and chronic cardiac remodeling post-infarction. A fast induction of cytokines (TNF- α , IL-6, IL-1 β) occurs as a response of mechanical and oxidative stress and hypoxia during ischemia. These cytokines have a pleiotropic effect on host cells which can result in either apoptosis or preservation and hypertrophy of cardiac myocytes. Besides, transmigration of macrophages and neutrophils contribute to the effect by amplifying these cytokines. A sustained upregulation of these cytokines or a second wave of cytokine upregulation in the heart are signs of chronic cardiac remodeling²⁸⁹. The increased cytokine expression causes an elevated activity of the local matrix metalloproteinases (MMPs) as well as an elevation of collagen

expression in the non-infarcted myocardium³⁴⁰. MMPs and their inhibitor, the tissue inhibitor matrix metalloproteinases (TIMPs), play an important role in the regulation of cardiac remodeling and angiogenesis³⁴¹ by influencing extracellular matrix (ECM) homeostasis. The cardiac ECM is comprised of fibrillar collagen types I and III³⁴². The excessive production and accumulation of ECM leads to increased stiffness of the myocardium and hinders ventricular contraction and relaxation, resulting in deformed architecture and disturbed function of the heart^{342,343}.

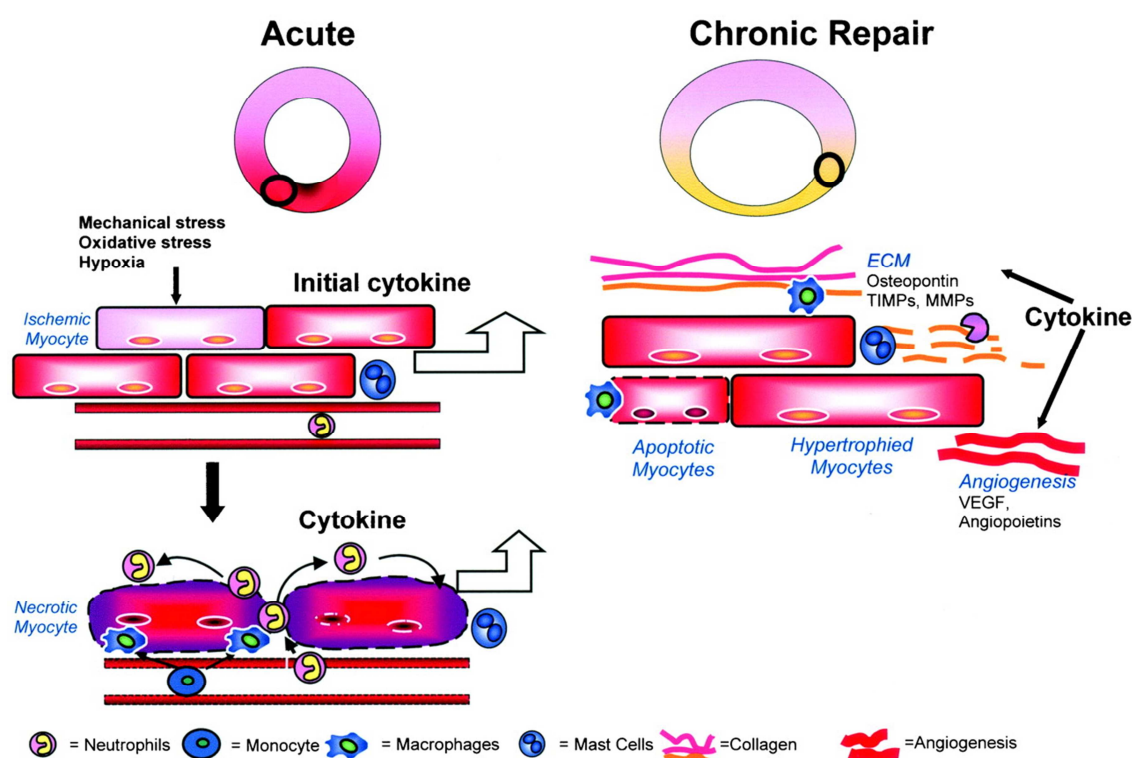


Figure 59. The induction of inflammatory cytokines after myocardial ischemia and their effect on acute and chronic cardiac remodeling post-infarction (Nian M et al. *Circulation Research* **94**, 1543-1553 (2004)) MMPs indicate matrix metalloproteinases; TIMPs, tissue inhibitor matrix metalloproteinases; ECM, extracellular matrix; VEGF, vascular endothelial growth factor.

Bioactive constituents of SNT and its known pharmacological activities

According to Tan et al. the major constituents of SNT are the *Aconitum* alkaloids, gingerols, flavonoids and saponins^{35,344}. In the present study liquiritin, isoliquiritin, liquiritigenin,

glycyrrhizic acid and eugenol were identified. In section 3.1.3 – 3.1.6 the pharmacological activities of each herb are described, implying that the SNT is a combination of herbs with multiple therapeutic effects. The cardio-active effect of C₁₉-diterpenoid alkaloids was observed in various studies. These include an improved inotropic effect and left ventricular diastolic function⁷⁶ assuming the administration of the optimal dose⁷⁵. Asl and Hosseinzadeh²⁶² report in their review on various cardiovascular effects of the saponins and flavonoids of *G. uralensis*. For example, glycyrrhizin possessed thrombin inhibition properties *in vitro* and *in vivo*^{215,345} and isoliquiritigenin showed vasorelaxant effects. It also decreased the tube formation in vascular endothelial cells³⁴⁶, this also being observed in the case of isoliquiritin³⁴⁶. In addition, *Z. officinale*, *C. cassia* and *G. uralensis* have an anti-inflammatory, an anti-oxidative and an anti-platelet aggregation effect in common. An extract of *Z. officinale* was found to inhibit the production of TNF- α and IL-1 β , among other markers, in lipopolysaccharide stimulated macrophages^{347,348}. It has been reported that gingerol, a bioactive compound of *Z. officinale*, showed positive inotropic effects and increased Ca²⁺ uptake in sarcoplasmic reticulum of human myocardium³⁴⁹. An extract of *G. uralensis* suppressed IL-6 and TNF- α production *in vivo*³⁵⁰. Liquiritigenin, a bioactive flavanone of *G. uralensis*, has been reported to significantly reduce the production of reactive oxygen species, TNF- α and IL-6³⁵¹. Eugenol reduced acute pain *in vivo* by the modulation of the glutamatergic and TNF- α pathway³⁵². The accumulation of these anti-inflammatory effects may be the reason for the remarkable reduction of inflammatory biomarker levels observed in the present study.

A general therapeutic effect is achieved by multi-constituents acting on several targets. Because of this, it is a great challenge to investigate TCM along the lines of western medicine. SNT has a long history of effective use in treating MI but, despite this, it is hard to evaluate the integral activity and to understand in detail its underlying mechanisms of action. To summarize, SNT showed in this study attenuating effects on collagen and inflammatory markers involved in post-MI response and an improvement of the heart function could be observed. Additional analysis is essential to not only gain further knowledge about SNT's mechanism of action but also to allot specific pharmacological activities to specific compounds.

Outlook

It would be interesting to analyze further the heart tissue of the rats and to conduct pathohistological and histochemical studies including connective tissue staining and quantification as well as the quantification of various markers such as collagen, MMPs and TIMPs. For the analysis of the expression of e.g. TNF- α , IL- β 1, hs-CRP, nuclear factor-kappa B (NF- κ B), MMP 2 and MMP 9 on protein and gene level, reverse transcriptase polymerase chain reaction and western blot analysis will be applied to gain more information on the underlying molecular mechanisms.

As previously mentioned, one of the next steps will be to attribute the specific pharmacological activities to the specific bioactive constituents of SNT. The *in vivo* experiment could be repeated under the same conditions and various bioactive constituents selected, such as the gingerols, shogaols, flavonoids and saponins, to test their influence on the inflammatory and collagen makers that have been analyzed in this study.

One consideration for the prospective research should be examining the combination of FPS and SNT. The treatment with FPS can be accompanied by adverse effects such as headache, nasopharyngitis, hyperkalemia, elevated creatine kinase and others³⁵³. The expectation is that the FPS dose could be reduced when combined with SNT and, as a consequence, the side effects could be reduced. Furthermore, an improvement of the patient's life quality is to be expected, in view of TCM's multi target oriented properties. Su et al. and Qin et al. observed a significant improvement on the life quality of patients treated with SNT^{59,60}.

Conclusion

To conclude, SNT significantly reduced the serum and cardiac tissue levels of collagen makers, col I and III, and the serum levels of inflammatory markers, IL-1 β , TNF- α and hs-CRP resulting in an attenuation of fibrosis and strong anti-inflammatory effects associated with the prevention of cardiac remodeling and, as a consequence, heart failure. In addition, it increased the serum level of the vascular active marker NO, attenuated post-MI ventricular dilation and maintained ventricular function. These results indicate the beneficial effects of SNT administration in rats' post-MI and in this way justify its traditional use in China. The poisonous aspects of

A. carmichaelii must be taken into account, screening methods for the toxic constituents improved and always applied prior to prescription. As the SNT consists of four herbs, no conclusions can be drawn at present about the bioactive components responsible for these effects.

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Abbreviations

A	aconitine
ADR	adriamycin
AMI	acute myocardial infarction
ANP	atrial natriuretic peptide
AP	angina pectoris
As	arsenic
AWT	anterior end-diastolic wall thickness
BCAA	branched-chain amino acid
BNP	brain natriuretic peptide
Ca ²⁺ ATPase	calcium adenosine triphosphatase
Cc	<i>Cinnamomum cassia</i>
Cd	cadmium
CHD	coronary heart disease
Col I	collagen type I
Col III	collagen III
CTNT	cardiac Troponin T
CVD	cardiovascular disease
DALY	disability adjusted life year
DDAs	diester diterpene alkaloids
D ₂ O	deuterated water, deuterium oxide
EF	ejection fraction
ELISA	enzyme-linked immunosorbent assay
ET-1	endothelin 1
EU	European Union
FAO	Food and Agriculture Organization
FPS	Fosinopril-Sodium group
FS	fractional shortening
Gu	<i>Glycyrrhiza uralensis</i>
HA	hypaconitine
Hg	mercury

HPLC-DAD	high-performance liquid chromatography diode array detector
hs-CRP	high sensitivity C-reactive protein
ICP-OES	inductively coupled plasma-optical emission spectroscopy
ID	isosorbide dinitrate
IL-6	interleukin-6
ICAM-1	intercellular adhesion molecule-1
i.p.	intraperitoneal
i.v.	intravenous
LAD	left anterior descending coronary artery
LOD	limit of detection
LOQ	limits of quantification
LV	left ventricle
LVDd	left ventricle end-diastolic dimension
LVDs	left ventricle end-systolic dimension
LVEDV	left ventricle end-diastolic volume
LVESV	left ventricle end-systolic volume
MA	mesaconitine
MET	myocardial endothelin
MeOD	deuterated methanol
MI	myocardial infarction
MMP	matrix metalloproteinase
Model	model group
Na ⁺ -K ⁺ ATPase	sodium-potassium adenosine triphosphatase
NF-κB	nuclear factor 'kappa-light-chain-enhancer' of activated B-cells
NMR	nuclear magnetic resonance
NO	total nitric oxide
Normal	normal group
Pb	lead
PKC	protein kinase C
p.o.	per oral
PTCA	percutaneous transluminal coronary angioplasty

PTWI	provisional tolerable weekly intake
PWT	posterior end-diastolic wall thickness
QC	quality control
R ²	correlation coefficient
ROESY	rotating-frame nuclear Overhauser effect spectroscopy
RSD	relative standard deviation
RT	retention time
SD	Sprague Dawley
SH-O	sham-operated group
Smac	second mitochondria-derived activator of caspase
SNT	“Si Ni Tang”
SNT-HD	Si Ni Tang-High Dose
SNT-LD	Si Ni Tang-Low Dose
SOD	superoxide dismutase
Std.dev.	standard deviation
TCM	traditional Chinese medicine
TGF-β1	transforming growth factor-β1
TNF-α	tumor necrosis factor-α
uAc	unprocessed <i>A. carmichaelii</i>
UV-VIS	ultraviolet-visible
WHO	World Health Organization
Zo	<i>Zingiber officinale</i>

List of figures

Figure 1. Components of SNT.....	12
Figure 2. Plant parts of <i>A. carmichaelii</i>	20
Figure 3. Chemical structure of aconitine, hypaconitine and mesaconitine.....	21
Figure 4. Plant parts of <i>Z. officinale</i>	23
Figure 5. Chemical structure of 8-Gingerol and 6-Shogaol.....	24
Figure 6. Plant parts of <i>C. cassia</i>	26
Figure 7. Chemical structure of eugenol, cinnamaldehyde and 4-methoxy-cinnamaldehyde...	27
Figure 8. Plant parts of <i>G. uralensis</i>	28
Figure 9. Chemical structure of liquiritin, isoliquiritin, liquiritigenin, isoliquiritigenin, licochalcone A and glycyrrhizic acid.....	29
Figure 10. Chemical structure of aconitine, hypaconitine and mesaconitine.....	31
Figure 11. The detoxification of diterpene alkaloids by two-step hydrolysis.....	33
Figure 12. Chemical structure of aconitine and liquiritin.....	44
Figure 13. Concentration of arsenic, lead, cadmium and mercury analyzed in twenty-four batches of medicinal plants.....	54
Figure 14. HPLC fingerprint profile of SNT.....	57
Figure 15. HPLC profile of standard solution VDR1 (I), decoction of unprocessed <i>A. carmichaelii</i> (II) and processed <i>A. carmichaelii</i> (III).....	65
Figure 16. HPLC profile of standard solution VDR1 (I) and HPLC fingerprint profile of SNT decoction with unprocessed <i>A. carmichaelii</i> (II).....	66
Figure 17. Section of HPLC profiles of the mixtures: standard solution VDR1 with bioactive compounds of <i>Z. officinale</i>	70
Figure 18. Section of HPLC profile of the combination of standard solution VDR1 with bioactive compounds of <i>G. uralensis</i>	72
Figure 19. Potency of isoliquiritin to reduce the amount of AC, HA and MA.....	74
Figure 20. What happens to MA, HA and AC in the mixture with isoliquiritin?.....	75

Figure 21. Corresponding UV-spectra of figure 20 of AC, HA and MA, isoliquiritin and the product of the aconitines with isoliquiritin.....	76
Figure 22. Chemical structure and ^1H -NMR spectra of aconitine in $\text{D}_2\text{O}/\text{MeOD}$ (2:1).....	77
Figure 23. Chemical structure and ^1H -NMR spectra of liquiritin in $\text{D}_2\text{O}/\text{MeOD}$ (2:1).....	79
Figure 24. Job's plot of the chemical shift changes of H-6 of liquiritin ($\delta = 6.44$ ppm) for the aconitine/liquiritin complex in $\text{D}_2\text{O}/\text{MeOD}$ (2:1).....	80
Figure 25. Section of ^1H -NMR titration spectra demonstrating the chemical shifts of H-6, H-8 and H-2 of free liquiritin in $\text{D}_2\text{O}/\text{MeOD}$ (2:1).....	82
Figure 26. Titration curve of aconitine/liquiritin.....	83
Figure 27. Section of titration curve of aconitine/liquiritin.....	84
Figure 28. Section of ^1H -NMR titration spectra demonstrating the chemical shift of H-12 of free aconitine in $\text{D}_2\text{O}/\text{MeOD}$ (1:2).....	86
Figure 29. Titration curve of liquiritin/aconitine.....	87
Figure 30. 2D ROESY spectra of a 1:1 mixture of aconitine (0.25 mM) and liquiritin (0.25 mM) in $\text{D}_2\text{O}/\text{MeOD}$ (2:1).....	88
Figure 31. Section of the 2D ROESY spectra of a 1:1 mixture of aconitine (0.25 mM) and liquiritin (0.25 mM) in $\text{D}_2\text{O}/\text{MeOD}$ (2:1).....	89
Figure 32. UV spectra of a constant concentration of liquiritin in absence and presence of different concentrations of aconitine.....	91
Figure 33. UV spectra shown in figure 32 combined in one graph.....	92
Figure 34. UV spectra of a constant concentration of aconitine in absence and presence of different concentrations of liquiritin.....	93
Figure 35. UV spectra shown in figure 34 combined in one graph.....	94
Figure 36. Effect of the treatment with SNT for 30 days on the body weight in myocardial-infarct rats.....	98
Figure 37. Effect of 30 days' treatment with SNT on the heart/body weight ratio.....	99
Figure 38. Effects of the treatment with SNT on the echocardiographic findings.....	101
Figure 39. Effect of the treatment with SNT on the LVDd, LVDs and EF.....	102

Figure 40. Col I in serum $\mu\text{g/L}$	105
Figure 41. Col I in tissue $\mu\text{g/g}$	107
Figure 42. Col III in serum $\mu\text{g/L}$	109
Figure 43. Col III in tissue $\mu\text{g/g}$	111
Figure 44. TGF- β 1 in serum ng/L	113
Figure 45. TGF- β 1 in tissue ng/g	115
Figure 46. ANP in serum ng/L	118
Figure 47. CTnT in serum $\mu\text{g/L}$	120
Figure 48. IL-6 in serum ng/L	123
Figure 49. IL-1 β in serum ng/L	125
Figure 50. IL-1 β in tissue ng/g	127
Figure 51. TNF- α in serum ng/L	129
Figure 52. hs-CRP in serum mg/L	131
Figure 53. ET-1 in serum ng/L	134
Figure 54. NO in serum $\mu\text{mol/L}$	136
Figure 55. Effects of the treatment with SNT on the cardiac structure in rats.....	138
Figure 56. Photomicrographs of the heart ventricles.....	139
Figure 57. Hematoxylin and eosin stained liver sections.....	141
Figure 58. HPLC profile of the established method.....	145
Figure 59. The induction of inflammatory cytokines after myocardial ischemia and their effect on acute and chronic cardiac remodeling post-infarction (Nian M et al. Circulation Research 94 , 1543-1553 (2004)).....	157

List of tables

Table 1. SNT is composed of parts of three plants and modified by the addition of the bark of <i>C. cassia</i>	12
Table 2. The following parameters were used for the microwave reaction system.....	37
Table 3. The gradient elution of the mobile phase used for the separation of the alkaloids.....	40
Table 4. The gradient elution of the mobile phase used for the analysis of SNT decoction.....	40
Table 5. Concentration [$\mu\text{g/mL}$] of the calibration standards for aconitine, hypaconitine and mesaconitine.....	41
Table 6. Combinations of herbs for decoction to analyze possible herbal interaction.....	42
Table 7. Concentration of aconitine and liquiritin for Job's plot experiment.....	44
Table 8. The six experimental groups for examining the effects of SNT on early ventricular remodeling in rats post- myocardial infarction <i>in vivo</i>	49
Table 9. Concentration of arsenic, lead, cadmium and mercury in the dried, grinded medicinal herbs.....	53
Table 10. Composition of the highest amount of each heavy metal concentration detected within one herbal group for the calculation of the weekly intake of SNT decoction.....	55
Table 11. Case example: calculation of the weekly intake of SNT decoction, according to the average dose for a 60 kg person in China using the concentration shown in table 10.....	55
Table 12. Linear regression equation and linear range.....	59
Table 13. Repeatability of the developed HPLC method for the determination of HA extracted with MeOH:H ₂ O (1:1) and recovery of AC, HA and MA in an unprocessed <i>A. carmichaelii</i> sample extracted with MeOH by adding 15.5 μg AC, 28.0 μg MA and 102.0 μg HA.....	59
Table 14. Intra-day and inter-day precision of the developed HPLC method for the determination of HA in an unprocessed <i>Aconitum</i> sample extracted with MeOH:H ₂ O (1:1)....	60
Table 15. Content of AC, HA and MA in eight processed and one unprocessed <i>A. carmichaelii</i> batches.....	61

Table 16. Concentration of HA obtained by the methanolic extraction compared to that determined by the aqueous extraction (decoction) of processed and unprocessed <i>A. carmichaelii</i> roots and the decoction of SNT, containing the same batch of unprocessed root of <i>A. carmichaelii</i>	63
Table 17. Combinations of the herbs for the decoction to analyze possible herbal interactions.....	67
Table 18. Concentration of HA in single unprocessed <i>A. carmichaelii</i> decoction compared to that of herbal combinations.....	68
Table 19. ¹ H-NMR spectra of aconitine in D ₂ O/MeOD (2:1).....	78
Table 20. ¹ H-NMR spectra of liquiritin in D ₂ O/MeOD (2:1).....	79
Table 21. Chemical shift changes of H-6 of liquiritin were observed and multiplied with the mole fraction of liquiritin.....	80
Table 22. Shift changes of H-8 (6.23 ppm), H-6 (6.44 ppm) and H-2 (5.42 ppm) of liquiritin at a concentration of 0.2 mM/L in D ₂ O/MeOD (2:1).....	84
Table 23. Shift changes of H-12 (2.87ppm) and H-15 (4.42 ppm) of aconitine at a concentration of 0.2 mM/L in D ₂ O/MeOD (1:2).....	85
Table 24. The six experimental groups for examining the effects of SNT on early ventricular remodeling in rats post-myocardial infarction <i>in vivo</i>	96
Table 25. Effect of the treatment with SNT for 30 days on the body weight in myocardial-infarct rats.....	97
Table 26. Effect of the treatment with SNT for 30 days on the heart/body weight ratio.....	100
Table 27. Effect of the treatment with SNT on the LVDd, LVDs and EF.....	102
Table 28. Col I in serum μg/L.....	106
Table 29. Evaluation of the significant value from multiple group comparison of col I level in serum.....	106
Table 30. Col I in tissue μg/g.....	108
Table 31. Evaluation of the significant value from multiple group comparison of col I level in tissue.....	108

Table 32. Col III in serum $\mu\text{g/L}$	110
Table 33. Evaluation of the significant value from multiple group comparison of col III level in serum.....	110
Table 34. Col III in tissue $\mu\text{g/g}$	112
Table 35. Evaluation of the significant value from multiple group comparison of col III level in tissue.....	112
Table 36. TGF- β 1 in serum ng/L	114
Table 37. Evaluation of the significant value from multiple group comparison of TGF- β 1 level in serum.....	114
Table 38. TGF- β 1 in tissue ng/g	116
Table 39. Evaluation of the significant value from multiple group comparison of TGF- β 1 level in tissue.....	116
Table 40. ANP in serum ng/L	119
Table 41. Evaluation of the significant value from multiple group comparison of ANP level in serum.....	119
Table 42. CTnT in serum $\mu\text{g/L}$	121
Table 43. Evaluation of the significant value from multiple group comparison of cTnT level in serum.....	121
Table 44. IL-6 in serum ng/L	124
Table 45. Evaluation of the significant value from multiple group comparison of IL-6 level in serum.....	124
Table 46. IL-1 β in serum ng/L	126
Table 47. Evaluation of the significant value from multiple group comparison of IL-1 β level in serum.....	126
Table 48. IL-1 β in tissue ng/g	128
Table 49. Evaluation of the significant value from multiple group comparison of IL-1 β level in tissue.....	128

Table 50. TNF- α in serum ng/L.....	130
Table 51. Evaluation of the significant value from multiple group comparison of TNF- α level in serum.....	130
Table 52. hs-CRP in serum mg/L.....	132
Table 53. Evaluation of the significant value from multiple group comparison of hs-CRP in serum.....	132
Table 54. ET-1 in serum ng/L.....	135
Table 55. Evaluation of the significant value from multiple group comparison of ET-1 in serum.....	135
Table 56. NO in serum μ mol/L.....	137
Table 57. Evaluation of the significant value from multiple group comparison of NO in serum.....	137

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