

***Illicium verum* Hook. f. and *Illicium anisatum* L.:** **Anatomical Characters and their Value for Differentiation**

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Abstract

In the last years intoxications after consumption of *Illicium verum* Hook. f. have been reported due to adulterations with *I. anisatum* L.. Many efforts have already been made to develop a simple method which would enable the detection of *I. anisatum* in powdered samples of *I. verum*. Since many controversial facts concerning the value of anatomic characters for differentiation have been published 13 botanically vouchered samples were re-examined by means of light microscopy, fluorescence microscopy, SEM, and confocal laser scanning microscopy.

All anatomical details show a great variability even between the follicles of a certain aggregate. This is also true for the cuticular striation which has been proposed in literature as reliable character. Special attention was paid to the structure of the aleurone grains of the endosperm and the shape of the calcium oxalate crystals in the testa. In contrast to previous assumptions these characters do not allow a certain differentiation either. However, the presence of hexagonal crystals can serve as indicator for samples of *I. anisatum*.

At present a reliable proof of purity of powdered samples of *I. verum* seems to be possible by chemical analytical methods only.

Key words

Illicium verum • *Illicium anisatum* • differentiation • microscopy • anatomical characterization

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Introduction

Illicium verum Hook. f. (Chinese star anise) is well known for its use as aromatic spice and its use in phytotherapy for the treatment of dyspeptic complaints and catarrhs of the respiratory tract [1]. However, in the last years intoxications after consumption of Chinese star anise have been reported due to adulterations of *I. verum* with the morphologically similar Japanese star anise (*I. anisatum* L.) containing anisatin and other related toxic sesquiterpene lactones [2].

Moreover recently a pharmaceutical company was supposed to have bought up about 90% of the world wide harvest of *I. verum* for the isolation of shikimic acid as starting material for the synthesis of Oseltamivir [3]. The resulting shortage raised concerns about the purity of the remaining herbal material. Chemical analytical methods like GC/MS / HPLC-MS or TLC [4, 5, 6, 7] are very sensitive. However, the quick sample preparation for microscopy work urged scientists since decades to find in addition reliable anatomical characters (Table 1). Zänglein *et al.* [12] evaluated critically most of the proposed characters and tried to find new ones. Only the shape and size of the sclereids in the columella remained as valuable features:

I. verum contains - besides simple formed sclereids - highly branched astrosclereids with a size up to 440µm, whereas with *I. anisatum* only in exceptional cases astrosclereids can be found, usually there are just simple formed sclereids (up to 320µm). Therefore the differentiation between the two species is possible but only in consideration of the size of the sclereids when the whole fruits are available.

In a recent publication [6] once more microscopic characters are presented for differentiation between the two species. We tried to assess the proposed characters on the basis of well documented plant material using different microscopic techniques (light microscopy, fluorescence microscopy, SEM, confocal laser scanning microscopy). Special emphasis is laid on the structure of the cuticle, the shape of calcium oxalate crystals and the structure of the aleurone grains.

Tab. 1. Microscopic characters under discussion for differentiation between *I. verum* and *I. anisatum*

Cuticular striation of the exocarp	<i>I. verum</i> : more developed and more raised than the cuticle striation of <i>I. anisatum</i> [8, 9]
Colour of fluorescence of the outer epidermis	<i>I. verum</i> : red – brown, <i>I. anisatum</i> : yellow [10]
Palisade cells of the endocarp	Size of the palisades of the endocarp (<i>I. verum</i> : 360 - 616 µm, <i>I. anisatum</i> : up to 400 µm); place of the biggest palisades of one seed (<i>I. verum</i> : near the margin where the fruit splits apart, <i>I. anisatum</i> : side opposite the margin); changeover of the thin – walled palisades to the sclereidal cells of the endocarp (<i>I. verum</i> : gradually, <i>I. anisatum</i> : abrupt) [8, 9, 11]
Epidermal cells of the testa	Shape of the cells and the number and size of the piths differ [8]
Sclereidal cells beneath the palisade cells of the testa	Shape of the cells and the intercellulars differ [8]
Aleuron grains	Quantity (<i>I. verum</i> less than <i>I. anisatum</i>); shape (<i>I. verum</i> : round, <i>I. anisatum</i> : elongated); surface structure (<i>I. verum</i> : rough-textured, inclusions badly seen, <i>I. anisatum</i> : smooth and shiny, inclusions well seen) [8,9]
Color of fluorescence of endosperm	<i>I. verum</i> : blue, <i>I. anisatum</i> : brown [10]
Sclereids in the columella	Astrosclereids (<i>I. verum</i>) / simple sclereids (<i>I. anisatum</i>) [8, 9]

Methods

Reference Material

Botanically authenticated samples of *Illicium verum* (7 samples) and *I. anisatum* (2 samples) were provided by the American Herbal Pharmacopoeia (AHP). Herbarium specimens and retention samples are archived at the AHP (Tab. 2). They originate from different locations in Japan, China, Taiwan and Vietnam. Two samples of *I. anisatum* were obtained from Japan via Prof. Jakkapan Sirithunyalug, Department of Pharmaceutical Sciences, Chiang Mai University and

for one other sample of *I. anisatum* we couldn't find out the origin. One further sample of *I. verum* was a commercially traded product (Mag. Kottas, Austria). The identities of all the samples used in the investigations were proofed by GC-MS.

Tab. 2. Sample numbers, sources and origins of the plant material. AHP #: voucher number of the American Herbal Pharmacopoeia

Species	Sample Number	Origin
<i>I. anisatum</i>	AHP #1014	Tokyo, Japan
<i>I. anisatum</i>	AHP #500b	Taiwan
<i>I. anisatum</i>	200501	unknown
<i>I. anisatum</i>	03/1	Tatayama, Japan
<i>I. anisatum</i>	03/2	Tatayama, Japan
<i>I. verum</i>	AHP #501	Vietnam
<i>I. verum</i>	AHP #502	China
<i>I. verum</i>	AHP #503	Vietnam
<i>I. verum</i>	AHP #508	Hänseler AG #9-3114-0
<i>I. verum</i>	AHP #524	China
<i>I. verum</i>	AHP #497	China
<i>I. verum</i>	451	unknown
<i>I. verum</i>	commercially traded product	Mag. Kottas, Austria

Technical Equipment and Sample Preparation

(a) *Light microscopy.* – Nikon Optiphot–2 microscope equipped with Samsung Digimax V50 Digital Camera; fragments (paradermal sections, transverse sections) of the follicles and seeds were placed on a glass slide, embedded in few drops of a solution of chloral hydrate (60% in water) and shortly boiled in order to clear the samples.

For the examination of the aleuron grains the samples were embedded in glycerine without boiling.

(b) *Phase contrast microscopy.* – Nikon Optiphot–2 microscope equipped with Samsung Digimax V50 Digital Camera. For the isolation of the cuticle paradermal sections of the follicle were placed into a saturated solution of chromium(VI)oxide in nitric acid (conc.). After three hours all structures except the cuticle were destroyed,

the cuticle was placed on a glass slide and after adding a solution of chloral hydrate (60% in water) the samples were examined under the microscope.

(c) *Fluorescence microscopy*. – Nikon Eclipse E 600 fluorescence microscope equipped with UV Ex filter 330 – 380. Fragments of the follicles as well as powdered plant material were examined dry as well as embedded in few drops of ethanol.

(d) *Scanning electron microscopy (SEM)*. – *Sputter-coater*. - Edwards Scancoat SIX , Phillips XL 30 ESEM – microscope. The dried follicles were sputter-coated with gold and examined using the HiVac - modus (secondary electron detector, acceleration voltage 15 kV).

(e) *Laser scanning microscopy (LSM)*. – Zeiss LSM 410 invert. The endosperm of the seeds was stained with acridine – orange, samples were mounted in water.

(f) *Gas Chromatography / Mass Spectrometry* – the identity of all samples was proofed using GC/MS of a dichloromethane extract of the freshly powdered material (parameters taken from lit. 6).

Results and Discussion

Cuticular striation

In order to get information about the structure of the cuticle from all parts of the exocarp we examined the surface using SEM. In addition we isolated the cuticle from paradermal sections from all parts of a follicle for phase contrast microscopy.

The surface of the follicles is considerably wrinkled, only except the area where the follicles touch each other. Therefore paradermal sections without further processing will provide only poor information due to the non planar surface. The pattern of the cuticular striation is not uniform within a certain follicle independent of the species or the source of the material. Similarities within a follicetum are restricted to the planar areas, however, no homogeneity could be found for several fruit aggregates of the same origin. The comparison of the cuticle at the planar areas of the two species showed a tendency for a more regular parallel striation in

I. verum and a more star-like pattern for *I. anisatum* (Fig. 1, 2, 3). A star-like pattern of the cuticular striation can only serve as a hint for *I. anisatum*. In contrast to the findings of Joshi *et al.* [6] the structure of the cuticle of the present plant material is too variable and cannot serve as a reliable character for differentiation as other patterns like parallel striation can also occur within this species.

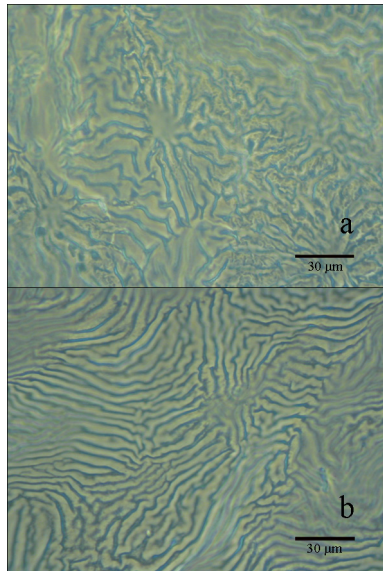


Fig. 1. Cuticular striation of the isolated cuticle under phase contrast microscopy of *I. verum* (a) and *I. anisatum* (b)

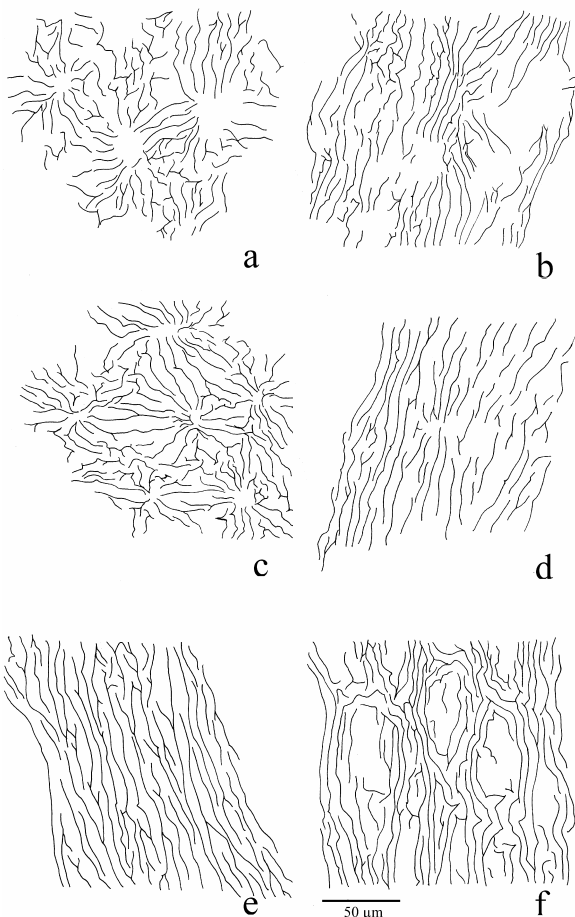


Fig. 2. Comparison of patterns of cuticular striation of *I. anisatum* (a, b) and *I. verum* (c–f)

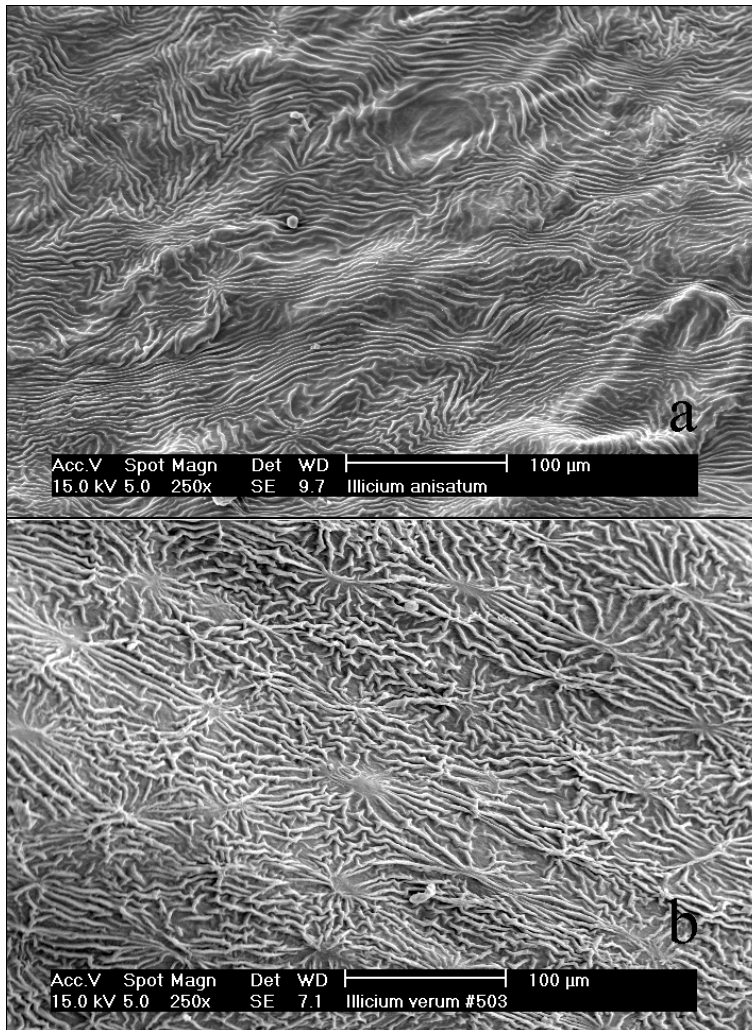


Fig. 3. Cuticular striation under SEM of *I. anisatum* (a) and *I. verum* (b)

Fluorescence

Contrary to Joshi *et al.* [6] and in accordance with Zänglein *et al.* [12] this study could not state different colours of fluorescence concerning the cuticle or the endosperm. The cells appear yellow to brown or blue. The intensity of the colours varies within the different samples of the same species.

Aleuron grains

Differences concerning the quantity of the grains cannot be confirmed as stated in literature [8]. The shape and surface structure the aleuron grains were examined by the means of light microscopy (Fig. 4) and SEM. The shape differs within the species from elongated to roundish which is in accordance to former studies [12]. In contradiction to literature [8, 9, 12] we found a similar variability of

the surface structure. The surface of the grains of *I. verum* and *I. anisatum* appears both smooth and rough depending on the sample probably due to possible pretreatments of the fruits as already mentioned [12].

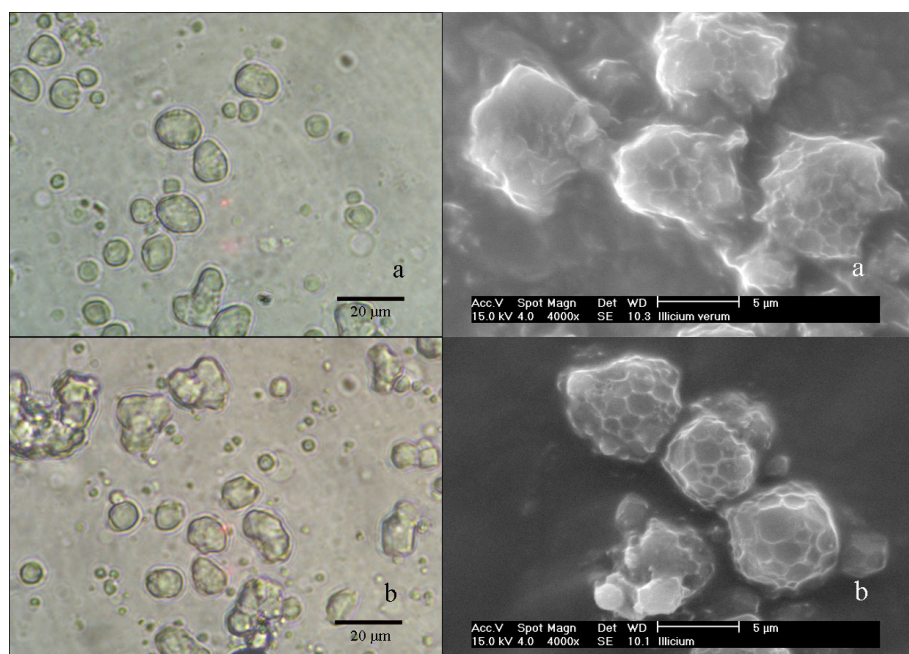


Fig. 4. Aleuron grains under light microscopy and SEM of *I. verum* (a) and *I. anisatum* (b)

The resolution of the light microscope is too low to allow an investigation of the inclusions of the aleuron grains. Therefore laser scanning microscopy (LSM) has been used which allows a distinct view at the number and dimension of possible inclusions. Optical sections at different focal planes revealed that there is a wide range in the quantity and size of the inclusions in both species. Inclusions may be absent as well as numerous in the aleuron grains of a certain seed independent of the *Illicium*-species. The size of the aleurone grains differs also in a high degree, no species-specificity could be found.

Calcium oxalate crystals

The layer between the brown parenchyma of the testa and the endosperm contains calcium oxalate crystals of different shapes and sizes – rectangular square, rhomboid, acuminate and hexagonal from 6 up to 54 µm in length (Fig. 5, 6).

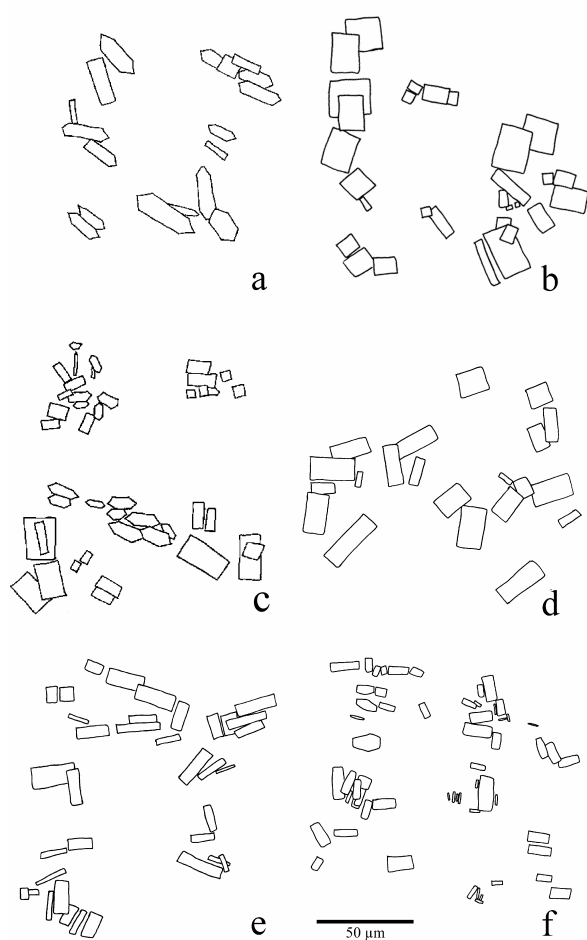


Fig. 5. Calcium oxalate crystals of *I. anisatum* (a–c) and *I. verum* (d–f)

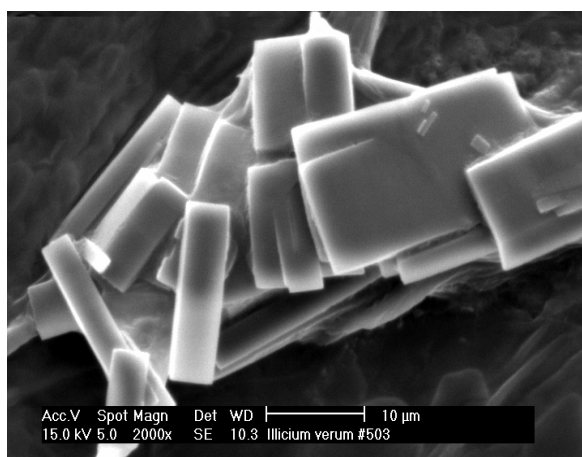


Fig. 6. Calcium oxalate crystals of *I. verum* under SEM

Although the size of the crystals is very homogenous within a certain seed large differences are possible even within a follicetum, the size of the crystals is not an indicator for one of the two species.

Large crystals with a hexagonal shape occur very frequently in the testa of *I. anisatum* while they are present very rarely in *I. verum*. Therefore the presence of hexagonal crystals in powdered material can be interpreted as an indicator for the presence of *I. anisatum*, the absence of hexagonal crystals can not be interpreted as a lack of *I. anisatum*.

Palisade cells of the endocarp

Our investigations confirm the findings in literature [12], there is no differentiation possible by the size of the palisades of the endocarp (as they overlap - *I. verum*: 360 - 616 µm, *I. anisatum*: up to 400 µm), by the place of the largest palisades of one seed (as that can be different depending on the sample) or by the changeover of the palisades to the sclereidal epidermal cells. In both species this change can happen both gradually and abrupt.

Epidermal cells of the testa and sclereidal cells beneath them

Neither the shape of the epidermal cells and the sclereidal cells nor the piths or the intercellular spaces can serve as differentiation characters. There is too much variability even within each species (compare Zänglein *et al.* [12]).

Astrosclereids

In accordance with Zänglein *et al.* [12] this study confirms the difference between *I. verum* and *I. anisatum* concerning the sclereids of the columella. Besides the simple formed sclereids that appear in both of the species, *I. verum* contains highly branched astrosclereids with a size up to 440 µm. The sclereidal cells of *I. anisatum* just reach a size up to 320 µm and astrosclereids are only rarely found. Hence, this character allows the only distinct differentiation of the both drugs by microscopic means as long as the whole fruits are available. It does not allow an identification of an adulteration with *I. anisatum* in powdered material.

Conclusions

Our observations show the heterogeneity of the plant material and the importance of a big number of different samples of different origins.

On the basis of these results, the best characters for a differentiation between *I. verum* and *I. anisatum* when using microscopy remain the shape and size of the sclereids in the columella. In whole fruits the typical astrosclereids of *I. verum* can easily be found in longitudinal sections of the columella. The possibilities for the microscopic detection of *I. anisatum* in powdered material are very limited. The structure of the cuticular striation, the size and shape of the aleurone grains as well as the colour of the fluorescence light are highly variable.

The cuticular striation of the exocarp of the fruit as well as the calcium oxalate crystals of the testa can only provide hints for the assessment of samples. Chemical analytical methods are first choice in the proof of the purity of samples of *I. verum*.

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