Detection of *Nicotiana tabacum* Leaf Contamination in Pharmaceutical Products

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*Nicotiana tabacum* (Solanaceae) is the only species whose leaves can be legally marketed as tobacco according to the Japanese Tobacco Business Act. Nicotine, a major alkaloid produced by *N. tabacum* leaves, is regulated in pharmaceuticals by the Japanese Pharmaceutical Affairs Law. However, the use of *N. tabacum* stems as an excipient in pharmaceuticals is permitted, because these contained only a small amount of nicotine. Recently, several reports showed that a substantial amount of nicotine was detected in an OTC pharmaceutical product, in which *N. tabacum* stems were used as an excipient. Therefore, products containing *N. tabacum* stems could be contaminated with the leaf material. In the present study, we established a method to detect contamination of *N. tabacum* stem materials with its leaves, using microscopy to obtain standard reference microphotographs for identification. Cultivated *N. tabacum* stems and leaves, commercial cigarette leaves, and *N. tabacum* tissue imported as excipient material were used for preparing the microphotographs. The characteristic *N. tabacum* leaf structures found in the powdered fragments included: epidermal cells with sinuous anticlinal cell walls, hairs, mesophyll parenchyma with crystalized calcium oxalate (calciphytoliths), and branching vascular bundles derived from reticulate net-veins. A comparison of the microscopic characteristics of an OTC powder with those from the standard reference microphotographs was an effective method for *N. tabacum* stem and leaf identification. Thus, we evaluated the powdered pharmaceutical product containing *N. tabacum* stem tissue and *Hydrangea serrata* (Hydrangeaceae) leaf tissue as excipients, and confirmed the presence of *N. tabacum* leaf material.

**Key words** microscopic morphology; *Nicotiana tabacum*; nicotine; pharmaceutical product; powdered preparation; Solanaceae

The Tobacco Business Act of Japan permits the use of *Nicotiana tabacum* (Solanaceae) leaves only for manufacturing tobacco products, including cigars, cigarettes, chewing tobacco, and snuff, and prohibits their use in other products. Nicotine, the major alkaloid in tobacco leaves, is classified as a poison by the Poisonous and Deleterious Substances Control Law in Japan, and its use in pharmaceutical products requires a permit, according to the Pharmaceutical Affairs Law in Japan. Conversely, the stem of *N. tabacum* contains low nicotine concentrations, thus, is used as an excipient. Recently, reports have shown that an OTC commercial pharmaceutical product contained a significant amount of nicotine, even though its approval document only listed the use of *N. tabacum* “stem peels” (SNT). This suggests that such products might contain *N. tabacum* leaves together with “stem peels”. The Pharmaceutical Affairs Law does not permit the use of ingredients not listed in the approval document; therefore, it is critical that we establish a rapid method to detect *N. tabacum* leaf contamination in excipients.

In the approval document, the excipients of the OTC pharmaceutical product are listed as SNT and *Hydrangea serrata* (Hydrangeaceae) leaves (LHS), which were fragmented in a similar manner to that used to prepare tobacco leaves in cigarettes. Thus, it is difficult to identify the origin of organ and tissue fragments by direct microscopic observation, and special sample preparation methods are required.

To prepare the samples for microscopic observations, the fragments should be pulverized into a “moderately fine powder” using a mortar and pestle. In the present study, we first elucidated the morphological and microscopic characteristics of the stem and leaf tissues of cultivated *N. tabacum*, leaf material collected from cigarettes, SNT imported from Indonesia and Thailand, and LHS. Subsequently, all samples were pulverized into “moderately fine powder” and the microscopic characteristics of each tissue was observed and recorded as standard reference microphotographs to determine the origins of the fragmented tissues. Using these microphotographs, the finely pulverized OTC pharmaceutical product, in which nicotine was detected by HPLC and MS, was analyzed by microscopy to detect the presence of *N. tabacum* leaf tissue.

**MATERIALS AND METHODS**

**Materials** Cultivated *N. tabacum* (*N. tabacum* L. cv. SR-1, NIH-DPP-20201) plant was maintained in sterilized shoot culture, acclimatized, and transferred to a pot in the Ozeki Laboratory (Department of Biotechnology and Life Science, Faculty of Engineering, Tokyo University of Agriculture and Technology), and cultivated in the 2nd laboratory of Division of Pharmacognosy, Phytochemistry and Narcot-
ics, National Institute of Health Sciences to maturity (Fig. 1a). The commercial brand “Caster Mild,” manufactured by Japan Tobacco Inc. was used as cigarette leaves. The remaining samples were provided by the Ministry of Health, Labour, and Welfare of Japan: the OTC commercial pharmaceutical product containing nicotine (CPN), and the raw materials of the CPN excipients, which included dried SNT (The label with description of used part was unclear. It is estimated the dermal layer of the stem by appearance) imported from Indonesia (NIHS-DPP-20202) and Thailand (NIHS-DPP-20203), and dried LHS (NIHS-DPP-20204) from cultivated plants at Ibaraki Prefecture, Japan. These samples are preserved at Division of Pharmacognosy, Phytochemistry and Narcotics of National Institute of Health Sciences. The CPN excipient percentages are 99% (SNT) and 1% (LHS) by w/w according to the directions.

Observations of the Morphological Characteristics  A digital microscope (VH-8000C; Keyence Co., Osaka, Japan) with a VH-Z25 zoom lens (Keyence) and unaided observations were used to conduct a morphological analysis of the
Observations of the Microscopic Characteristics All microscopic characteristics were observed by normal and polarized light using the following microscopes: BH-2 (Olympus Co., Tokyo, Japan) with an attached VH-8000C, and an Axio Scope A1 (Carl Zeiss AG, Oberkochen, Germany) with an attached DP-21 digital camera. Measurements were made using the measuring function of the digital microscope, and 30 or more measurements were made for each tissue type (10 or more in less frequent tissues).

For microphotography, a Micrograph Imager Olympus PM-10AK (Olympus Co.), VH-8000C, and DP-21 digital camera (Olympus Co.) were used.

Surface View and Transverse and Longitudinal Sections The fine-scale epidermal structure was observed using prepared specimens. The outermost layer of both stem and leaf were peeled, and dipped in a Petri dish filled with water. Then tissues were placed on a glass slide and the mixture of glycerol and water (1:1) as described in the “Microscopic examination” section of the 16th edition of the Japanese Pharmacopoeia (JP16) was added to the tissues. The samples were then covered with a cover glass.

Transverse and longitudinal sections were prepared by freeze sectioning. Rough sections, circa 3–5 mm thick, were cut using a razor blade, then frozen and sliced into 30–60-μm-thick sections using a freezing microtome (KELK Ltd., Kanagawa, Japan). The sections were then dipped in water, placed on glass slides, and mounted as described above.

For the observation of the mature leaf structures, we mainly used leaves from commercial tobacco (cigarettes). The dried materials, cigarettes, and SNT samples were soaked in water for 20–30 min at the ordinary temperature to restore cell shape. After this, the outermost layer and sections were prepared by the same technique as described above. Only one epidermal sample of LHS was prepared, because of the difficulty of sectioning LHS samples.

Pulverized Samples To identify the microscopic characteristics of tissue fragments, all samples, i.e., stem and leaf samples of cultivated N. tabacum were dried for more than three days at the ordinary temperature; commercial cigarette leaves, SNT, LHS, and CPN excipients were pulverized using a mortar and pestle. The powders were sieved through a #50 (nominal designation of sieve, 300 μm) sieve to obtain a “moderately fine powder” to “very fine powder”. According to the “Microscopic examination” section of JP16, circa 1 mg of powder was mixed with a drop of the mounting agent on a glass slide. Then, the mixture was stirred carefully with a small rod not to produce any air bubbles, and allowed to stand until rehydrated. Then, an additional drop of the mounting agent was added to the sample, and covered with a cover glass.

RESULTS AND DISCUSSION

Microscopic Characteristics of N. tabacum Leaf and Stem Tissues (Supplementary Fig. 1) We observed the microscopic characteristics of N. tabacum leaf and stem tissues of a plant maintained in a sterilized shoot culture, and cigarettes (Supplementary Fig. 1A). We found that bicollateral vascular bundles, which are specific to Solanaceae, glandular hairs, non-glandular multicellular hairs, and cells containing crystalized calcium oxalate (calciphytoliths) were characteristic features in N. tabacum stems and leaves. In the surface view of stems, oblong epidermal cells accompanied by the stomata were arranged longitudinally. In the transverse section of the stem, fiber bundles appeared at the outer side the external phloem. The cortical parenchyma cells, large vessels of approximately 30 μm in width (spiral, reticulated, and bordered pit vessels) and large parenchyma cells containing starch grains were long in the longitudinal section, which was characteristic (Supplementary Fig. 1B). In contrast, epidermal cells with sinuous anticlinal cell walls, accompanied by anomocytic stomata, branching vascular bundles, and mesophyll parenchyma cells (palisade and spongy tissues) containing chloroplasts and/or calciphytoliths, were observed only in leaves. Numerous calciphytoliths were observed in mature leaves. Characteristics of epidermal cells on the vein or petiole resembled those of stems (Supplementary Fig. 1C). These characteristics corresponded to the drawing of an N. tabacum leaf by Fujita and Esau.

Microscopic Characteristics of Powdered N. tabacum (Supplementary Fig. 2) The samples used in this investigation, i.e., CPN excipient and LHS, were difficult to prepare as sections because of their irregular fragmentation and fragility. Therefore, we examined the microscopic characteristics of these samples as a fine powder. Similarly, we examined the powder of dried cultivated N. tabacum stems and leaves and prepared standard reference microphotographs for identification.

In the powdered stem of cultivated plant (Supplementary Fig. 2A), fragments of longitudinal polygonal epidermal cells and stomata arranged lengthwise were observed. The fragments were occasionally accompanied by glandular hairs and/or stomata, but fragments of epidermis were not often observed in powders. Other major tissue fragments were: longitudinal cells, i.e., oblong cortical parenchyma cells, medulla parenchyma cells, fibers, vascular bundles, and glandular hairs. Some parenchyma cells contained calciphytoliths and/or starch grains. These characteristics corresponded to those obtained from the surface view, or a longitudinal section of the cultivated stem. In the mature stem powder, we observed large vessels, large fibers, and large parenchyma cells containing starch grains; these are discussed later under the SNT section (Fig. 1C).

In the powder of dried cultivated plant’s leaves and commercial cigarettes (Supplementary Fig. 2B), fragments of the epidermis of leaf blades, composed of epidermal cells with sinuous anticlinal cell walls and anomocytic stomata were observed at low frequencies. Sometimes these fragments accompanied glandular or non-glandular hairs. The features of the epidermal fragments derived from the leaf vascular bundles and petioles were similar to those of the stem epidermis; therefore, it was difficult to discern the two. Green or brown fragments derived from the mesophyll were frequently observed. In the dried cultivated young leaf powder, the mesophyll parenchyma (palisade and spongy tissues) specific to leaves included few calciphytoliths. However, in the cigarette leaf powder, numerous brown parenchyma fragments containing calciphytoliths were observed, which were characteristic of mature leaves. These calciphytoliths were visible under polarized light. Some vascular bundles in the mesophyll were branched, which were a characteristic trait of net-veined di-
Morphological and Microscopic Characteristics of Raw Materials of CPN Excipients

We observed the morphological characteristics of SNTs (Figs. 1A, 2A) imported from Indonesia (Figs. 1A-c, -d). Because the label with description of the used part of the SNTs was unclear; therefore, we first determined whether it was stem tissue or not. The SNTs from both Indonesia and Thailand were grayish-brown in color, and flat and string-like in shape. Their outer surfaces were covered with numerous hairs (Figs. 1A-a–d).

Thus, epidermal cells with sinuous anticlinal cell walls, mesophyll parenchyma cells containing calciphytoliths, and branching vascular bundles could be used to identify *N. tabacum* leaf tissue. Using these characteristics as indices, *N. tabacum* leaf tissue contamination could be detected in stem samples.

Morphological and Microscopic Characteristics of Attached Curled Thin Tissue of SNT

We observed the morphological characteristics of SNTs (Figs. 2A-a–c). Bar length: 1 cm. Microscopic characters (B): (a), (c) and (e), prepared from Indonesian SNT as shown in Figs. 2A(a); (b), (d) and (f), prepared from Thailand SNT as shown in Figs. 2A-b; (a) and (b), exfoliated epidermis; (c) to (f), transverse sections; (c), (d), and (e), overall view; (c) and (d), vascular bundle region; (e), mesophyll; (f), vascular bundle and calciphytoliths under light and dark fields. Arrows indicated epidermal cell at the base of a glandular hair. Bar length: (a), (b) and (f), 50 μm; (c) to (e), 200 μm. Microscopic characters of powder of curled thin tissue (C): (a) to (c), fragments of epidermis along with stoma; (d) and (e), mesophyll parenchyma; (f) to (i), vessels; (j) and (k), fragments of glandular hair. Epidermal cells in (a) to (c) had sinuous anticlinal cell wall. Mesophyll parenchyma in (d) and (e) sometimes contained calciphytoliths. Calciphytoliths in (e) shone under the dark field. Vessels in (f) to (i) were mainly observed as spiral ones. Vascular bundles were sometimes observed as branched ones as shown in (f). Bar length: 50 μm. cd: calciphytoliths; gh: glandular hair, mes: mesophyll, sto: stoma, vb: vascular bundle.
Sometimes curled, thin tissues were observed on the surface. The primary flat tissues of the SNT imported from Thailand were thinner than those from Indonesia. The inner surface of these tissues from Thailand was more fibrous than those from Indonesia. The darker colored portions of the samples imported from Indonesia were considered to be xylem. These observations suggested that both samples were dried tissues from the cortex of mature stems. When the curled thin tissues (Figs. 2A-a, -b) attached to the outer surface were soaked in water and spread, the typical net-veined pattern of dicotyle- donous plants was observed under a digital microscope (Fig. 2A-c). This suggests that the SNT might be contaminated with leaf material.

To observe the microscopic characteristics of the flat tissues considered to originate from the cortex (Fig. 1B), SNTs were soaked in water, and the outermost layer was peeled, after which transverse and longitudinal sections were prepared. The outermost layer in the surface view consisted of nearly colorless to slightly brown polygonal cells. Among the outermost cells, stomata were arranged regularly and longitudinally (Figs. 1B-a, -b), indicating that these were epidermis. The epidermal cells of the samples imported from Indonesia and Thailand were approximately 80 and 120 µm in diameter, respectively, and the stomata were 35–41–49 and 37–45–49 µm in diameter, respectively (Table 1). Glandular hairs with uniseriate stalks were scattered, and measured approximately 250–300 µm or above in length, and base of stalks were approximately 40 µm in diameter. Glandular cells were observed at the heads of those stalks. In the transverse section (Figs. 1B-c to -e), several layers of collenchyma cells were located directly inside the epidermis. In the samples from Thailand, the collenchyma cells were well developed and formed a thick layer (Fig. 1B-e). Adjacent to the collenchyma cells, cortical parenchyma cells, fiber bundles, and phloem were observed. Some cortical parenchyma cells contained primarily simple starch grains of 5–11 µm in diameter. Fiber bundles were comprised of small numbers of fibers, which were approximately 44 µm in diameter. In the samples imported from Indonesia, xylem was sometimes found adjacent to the phloem. In the samples imported from Thailand, phloem was rarely observed.

In the longitudinal section, most cells were vertical along their longitudinal axis, and some parenchyma cells in both Indonesian and Thailand samples contained calciphytoliths and/or starch grains (Figs. 1B-f, -g).

Based on these observations, the samples imported from Indonesia were mainly composed of tissues from the outer cambium of the bicollateral vascular bundle of the N. tabacum stem, whereas those imported from Thailand were composed mainly of epidermis and cortex of the stem.

When these tissues were powdered, fragments of the epidermis were not often observed, but they exhibited a characteristic longitudinal oblong shape, sometimes accompanied glandular hairs and/or stomata (Figs. 1C-a, -b). Other major tissue fragments that appeared in the powder were longitudinal cells, i.e., fragments of collenchyma cells (Fig. 1C-c), oblong cortical parenchyma cells (Fig. 1C-d), fibers (Figs. 1C-e, -f), some parenchyma cells containing starch grains and/or calciphytoliths (Figs. 1C-g, -h), and glandular hairs (Figs. 1C-i, -j). Fragments of bordered pit vessels and reticulate vessels were often observed (Figs. 1C-k, -l). These features corresponded to the microscopic characteristics of fragments of the pulverized cultivated and dried N. tabacum stem (Supplementary Fig. 2A).

When the curled tissues attached to the surface of the stem were observed (Figs. 2A-a, -b), almost colorless to slightly brown polygonal epidermal cells with sinuous anticlinal cell walls and randomly arranged anomocytic stomata were observed in the surface view (Figs. 2B-a, -b). This stomatal

Table 1. Morphological Comparison of Each Sample of N. tabacum and H. serrata

<table>
<thead>
<tr>
<th>Tissues</th>
<th>Samples</th>
<th>N. tabacum</th>
<th>H. serrata</th>
</tr>
</thead>
<tbody>
<tr>
<td>Shape of epidermal cell</td>
<td>Stem</td>
<td>Leaf (blade)</td>
<td>Leaf (blade)</td>
</tr>
<tr>
<td>Cultivar</td>
<td>Indonesia</td>
<td>Thailand</td>
<td>Cultivar</td>
</tr>
<tr>
<td>Major axis diameter&lt;sup&gt;a&lt;/sup&gt;</td>
<td>66–125</td>
<td>41–83</td>
<td>145</td>
</tr>
<tr>
<td>Minor axis diameter&lt;sup&gt;a&lt;/sup&gt;</td>
<td>21–31</td>
<td>24</td>
<td>40</td>
</tr>
<tr>
<td>Arrangement of stomata</td>
<td>Regularly longitudinal</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Major axis diameter&lt;sup&gt;a&lt;/sup&gt;</td>
<td>35–43</td>
<td>35–41</td>
<td>49</td>
</tr>
<tr>
<td>Minor axis diameter&lt;sup&gt;a&lt;/sup&gt;</td>
<td>23–30</td>
<td>24</td>
<td>31–38</td>
</tr>
<tr>
<td>Morphology of hair</td>
<td>Glandular hair</td>
<td>+ (250–300&lt;sub&gt;c&lt;/sub&gt; µm&lt;sup&gt;d&lt;/sup&gt; in length)</td>
<td></td>
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<tr>
<td></td>
<td>Non-glandular hair</td>
<td>Multicellular hair</td>
<td></td>
</tr>
<tr>
<td>Other characteristic tissues</td>
<td>Collechyma cell, Fiber (30 µm&lt;sup&gt;e&lt;/sup&gt;</td>
<td>44 µm&lt;sup&gt;f&lt;/sup&gt;), Vessel (30 µm&lt;sup&gt;e&lt;/sup&gt;), Single starch grain (5–8 µm&lt;sup&gt;e&lt;/sup&gt;</td>
<td></td>
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<td></td>
<td>Mesophyll cells, Calciphytoliths&lt;sup&gt;g&lt;/sup&gt;, Spiral vessel 5–9 µm&lt;sup&gt;g&lt;/sup&gt;</td>
<td></td>
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<td>Mesophyll cells, Needle crystal (22–40 µm, 70&lt;sub&gt;c&lt;/sub&gt; µm), Vessel (spiral 5–15 µm), (scalariform and pitted 10–16 µm)</td>
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</table>

<sup>a</sup> The values indicate minimum-average-maximum (µm).<sup>b</sup> Diameter of sinuous polygonal cells.<sup>c</sup> Diameter of polygonal cells.<sup>d</sup> Typical length of hair of sample from Indonesia.<sup>e</sup> Typical length.<sup>f</sup> Typical diameter of cultivar.<sup>g</sup> Typical diameter of tissues of sample from Indonesia.<sup>h</sup> Not detectable in cultivar immature leaves.
arrangement is typical of a dicotyledonous leaf and not observed in the stem epidermis. The epidermal cells from the Indonesian (Fig. 2B-a) and Thailand (Fig. 2B-b) samples were approximately 70 and 120 µm in diameter, respectively, and the stomata were 23–33–40 and 31–38–42 µm in diameter, respectively (Table 1). In both samples, the remaining circular traces of the fallen glandular hairs on the surface of the epidermal cells were observed in some epidermal cells and these epidermal cells did not differ in size or shape from other epidermal cells.

In the transverse section, epidermal cells were observed on both adaxial and abaxial sides of the leaf, along with scattered glandular hairs. A large vascular bundle was located in the enlarged portion, which was indicative of leaf vein tissue (Figs. 2B-c, -d). The vascular bundle was bicollateral. In mesophyll, palisade and spongy tissues were observed (Fig. 2B-e). Some cells contained calciophytoliths, which shone under polarized light (Fig. 2B-f). Lateral vascular bundles were observed in the transverse or longitudinal sections. Vessels or tracheids were primarily spiral in form, and 5–9 µm in diameter. Because the curled tissue in the samples imported from Thailand was very thin and fragile, it was difficult to distinguish both palisade and spongy tissues, but the vascular bundles of the leaf vein and calciphytoliths were clearly discernible (Figs. 2B-d, -f).

In the microscopic observations of the adjacent leaf tissue powder (Fig. 2C), the epidermis composed of epidermal cells with sinuous anticlinal cell walls and anomocytic stomata (Figs. 2C-a, -c) was not commonly observed. Numerous and distinctive tissue fragments derived from mesophyll tissue were fragmentary observed as a brown-colored mass. Sometimes mesophyll parenchyma cells accompanied vessels and/or a few calciophytoliths (Figs. 2C-d, -e). Fragments of vascular bundle in the mesophyll were sometimes branched (Fig. 2C-f). Spiral vessels were mainly observed in the vascular bundle (Figs. 2C-f to -i). Fragments of glandular hairs were also observed (Figs. 2C-j, -k). These features corresponded to those (Figs. 2C-f to -i). Fragments of glandular hairs were also observed (Figs. 2C-j, -k). These features corresponded to those of the pulverized leaf from a cultivated N. tabacum, and the cigarette sample (Supplementary Fig. 2).

From the observations of the main flat tissues from SNTs and the curled thin tissues attached to them, the two kinds of tissues were determined to be derived from N. tabacum stem and leaf, respectively. Because of the identifiable characteristics of both organs described above, it is possible to confirm contamination of leaf tissue in a stem sample at the raw material stage.

Morphological and Microscopic Characteristics of LHS (Fig. 3)

The morphological characteristics of another raw material of CPN excipient, i.e., dried LHS, were investigated. The dried LHS consisted of curled and fragile brown tissues, some of which were broken (Fig. 3A-a). When the samples were soaked in water and spread out, an oblong leaf with serrated leaf margin and cusp apex was observed. The adaxial and abaxial surfaces were light brown and slightly grayish brown, respectively. Numerous gently curved colorless hairs were observed on both surfaces. Net-veins were highly noticeable, and vascular bundles protruded from the abaxial side of the leaf (Fig. 3A-b).

In the surface view of the exfoliated epidermal tissues, they were accompanied by brownish mesophyll cells, and it was often difficult to determine the microscopic characteristics of epidermal cells and stomata (Fig. 3B). Epidermal cells were polygonal or sinuously polygonal in shape, measuring 25 and 56 µm in average diameter of major axis, respectively (Table 1). The epidermal cells over the vascular bundles were oblong, with some exceeding 100 µm in major axis diameter. The anomocytic stomata were nearly circular (Fig. 3B-a) measuring 15–21–26 µm in major axis diameter (Table 1). Unicellular hairs with sharp tips and scattered verrucous projections in the wall were also observed (Fig. 3B-b). The hairs were approximately 200–270 µm or more in length, and the base of the hair was approximately 35–50 µm in diameter. Epidermal cells around the base of the hair were arranged radially (Fig. 3B-b). These features of hairs were characteristics of LHS, which were distinguishable from SNT. The parenchyma cells of the mesophyll were brown and in a reticulate arrangement. Raphides of calcium oxalate were observed, measuring approximately 22–40 µm in length, with some exceeding 70 µm. Spiral vessels of 5–15 µm in diameter were observed in the vascular bundles (Fig. 3B-c).

In the microscopic observation of powdered LHS (Fig. 3C), fragments of epidermis accompanied by mesophyll tissue were clear to be brown in color. The epidermal cells were polygonal or sinuoso polygonal in shape, sometimes accompanied by stomata and/or warty unicellular hairs (Fig. 3C-c). The unicellular hairs with verrucous projections were characteristic of LHS (Fig. 3C-d). Radially arranged epidermal cells were located around the base of these hairs (Fig. 3C-e). Some fragments derived from the mesophyll contained brown substances (Fig. 3C-f). Several fibers in fragments were colorless, and approximately 15–20 µm in diameter. Some fiber bundles contained colored substances (Fig. 3C-g). Spiral, scalariform, and pitted vessels were clear to be brown in color (Figs. 3C-h–j) and the scalariform and pitted vessels were approximately 10–16 µm in diameters. The needle crystals appeared either as bundles (raphides) or were scattered (Fig. 3C-k).

Based on the above results, leaves of N. tabacum and H. serrata used as CPN excipients were distinguishable from each other by their differences in hair feature, crystal shape, vessel diameter, and features of the epidermal cells around the base of hairs.

Identification of Leaf Tissues in CPN (Fig. 4)

Using the index photographs of stem and leaf tissues of N. tabacum and leaf tissue of H. serrata summarized in Supplementary Fig. 2, Figs. 1 to 3 as references, we attempted to confirm that the CPN excipients contained N. tabacum leaf tissue. As shown in Fig. 4A, the CPN excipients were almost brown in color, and made of dried, irregularly cut pieces approximately 3 mm long and 0.3–0.5 mm wide. Therefore, their species and organ of origin were difficult to determine based on morphological characters alone (Fig. 4A). As the pieces were cut randomly, it was difficult to prepare a section after they were soaked in water. The CPN excipient was powdered using a mortar and pestle, and observed the microscopic characteristics of the “moderately fine powder” to “very fine powder.”

Overall, when the fragments of powdered CPN excipients were analyzed at low magnification, fibers and large parenchyma cells containing starch grains (Fig. 4B-a) were observed. Most fiber fragments were very long. Because the main raw materials originated from stems of N. tabacum, numerous
longitudinal tissue fragments were observed. The epidermal cells with sinuous anticlinal cell walls, therefore originating from leaf material, occurred infrequently, but were noticeable. Intact glandular hairs with uniseriate stalks, characteristic of *N. tabacum*, were rarely observed, but the fragments of wide stalk with thinner walls, and those of glandular cells, were often observed. Tissue fragments identified as *N. tabacum* stem (Fig. 4B shown as /uni25CF/) were large fibers (Figs. 4B-b, -c) and cortical parenchyma cells (Figs. 4B-d, -e). Some parenchyma cells contained calciphytoliths (Fig. 4B-d). Large parenchyma cells containing starch grains were also characteristic of the stems (Fig. 4B-e). Fragments of large bordered pit vessels were observed, suggesting that xylem tissue of *N. tabacum* was included in the material as an excipient (Figs. 4B-f, -g). Fragments of vessels with various thickened walls, *i.e.*, spiral, reticulate, scalariform vessels (Figs. 4B-h–j) were often observed. However, vessel fragments could not be used for identification of the organ of origin of *N. tabacum*.

Long epidermal cells with stomata distributed on the longitudinal axis were observed infrequently (Figs. 4B-k, -l). These features corresponded to the microscopic characteristics of the *N. tabacum* stem epidermis. Because the average diameter of stomata in *N. tabacum* stem was larger than 40 µm (Table 1), the stomata with diameter of longer than 40 µm appeared...
individually, and could be of the stem epidermis (Fig. 4B-m).

Moreover the tissue of LHS (Fig. 4B, shown as ○), composed of epidermal cells, accompanied by brown circular mesophyll cells containing raphides was also observed (Figs. 4B-n, -o). Radially arranged epidermal cells (Fig. 4B-p) exhibited the same shape as those around the bases of the warty unicellular hairs with verrucous projections (Fig. 4B-q). LHS tissue was mixed with the excipient at a concentration of only 1%, but the above fragments and cells were distinctive enough to allow for identification of LHS in the powdered excipient.

However, the fragments (Fig. 4B, shown as ○) derived from *N. tabacum* leaves were observed in the same preparation of powdered CPN as mentioned above, namely, as epidermal cells with sinuous anticlinal cell walls (Fig. 4B-r), anomocytic stomata, epidermal cell at the base of a hair (Fig. 4B-t), mesophyll parenchyma containing calciphytoliths, and branching vascular bundles (Fig. 4B-s); all of these were easily identified. These features are consistent with the typical
characteristics of \textit{N. tabacum} leaves, as shown in Supplementary Fig. 2.

Although glandular hairs, a specific characteristic of the Solanaceae family (Figs. 4B-u, -v), were often observed in powdered CPN excipients, the existence of leaf tissue could not be demonstrated by their detection, because they were found in both stem and leaf tissues.

CONCLUSION

The curled thin tissues attached to the surface of the SNT were considered to be \textit{N. tabacum} leaf tissue based on the following characteristics: 1. Epidermal cells with sinuouis anticlinal cell walls and anomocytic stomata observed on the epidermis, accompanied by glandular hairs covering both surface; 2. Large bicollateral vascular bundles in the projecting part, identifiable as leaf veins; 3. Palisade and spongy tissues, and calciphytoliths included in those tissues; and 4. Branching vascular bundles. These microscopic characteristics of \textit{N. tabacum} leaves were observed in the powdered samples. This demonstrated that the standard microphotographs of \textit{N. tabacum} tissue were useful for distinguishing leaf tissue from stem tissue, and the above characteristics could be used to successfully detect \textit{N. tabacum} leaf contamination in the stem tissues used as an excipient of pharmaceutical product. Furthermore, we investigated a current OTC commercial product and detected contamination by \textit{N. tabacum} leaf tissue, however, the level of contamination was very low (data not shown). This strongly suggests that the \textit{N. tabacum} leaf tissue was not added intentionally; processing methods for the production of the excipient material should be improved to avoid contamination.

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Supplementary Materials The online version of this article contains supplementary materials.

Supplementary Fig. 1. Material from cultivated \textit{Nicotiana tabacum} and commercial cigarette leaves used to compare the morphology between stems and leaves (A): (a), immature plant around 20 cm in height; (b), stem and petiole; (c), pieces of commercial cigarette leaf (mature leaf). Leaves in (a) were covered with numerous hairs, around 10 cm in length. Petiole in (b) was flat at the base and appearing to clasp the stem (shown as the arrow, decurrent). Bar length: (a), 5 cm; (b), 5 mm; (c), 1 mm. Microscopic characteristics of the stem of a cultivated \textit{N. tabacum} plant (B): (a) and (b), exfoliated epidermis; (a), epidermal cells and stoma; (b), glandular hair and multicellular hair; (c) to (f), transverse sections; (c), overall view; (d), fiber bundle; (e), bicollateral vascular bundle; (f), parenchyma cells of medulla with starch grains; (g) to (n), longitudinal sections; (g), epidermis with glandular hair; (h), fibers located outside of external phloem; (i), bordered pitted vessel with simple perforation; (j), reticulate vessel; (k), spiral vessel; (l), ring vessel; (m), spiral vessel and ring vessel; (n), parenchyma cells of medulla with starch grains. Bar length: (a), (b), (e) and (g), 50 µm; (c), 100 µm; (d), (f) and (h) to (n), 20 µm. Microscopic characteristics of the leaf of a cultivated \textit{N. tabacum} plant and cigarette leaves (C): (a) to (c), exfoliated epidermis of leaf blade of plant; (a), adaxial side; (b) and (c), abaxial side; (d), exfoliated epidermis of leaf blade of swelled cigarette leaves; (e) and (f) epidermis on the vein of swelled cigarette leaves; (g) and (h), exfoliated epidermis accompanied by glandular hairs and stomata on the plant leaf vein; (i), exfoliated epidermis of the petiole of the plant showed in Supplementary Fig. 1A-a and b; (j) to (o), transverse sections of leaf; (j), overall view of a mid-rib; (k), collenchyma cells; (l), bicollateral vascular bundle; (m), palisade and spongy tissues, and a lateral vascular bundle; (n), glandular hair with short stalk; (o), fiber bundle of cigarette leaves (matue leaves). Each arrow in (c) indicates the epidermal cell at the base of a glandular hair. Calciphytoliths in (f) shone under the dark (polarized) field. Bar length: 50 µm except for (e) and (j); (e), 100 µm; (j), 200 µm. co: collenchyma cell, cd: calciphytoliths, ep: epidermal cell, cx: cortex, fb: fiber bundle, gh: glandular hair, mh: multicellular hair, p: parenchyma cell, pa: palisade tissue, pfs: simple perforation, ph: phloem, sta: starch grains, stoma: stoma, vb: vascular bundle, vs: spiral vessel, x: xylem.

Supplementary Fig. 2. Fragments of powdered stems of a cultivated \textit{N. tabacum} plant (A): (a), epidermis consisted of long epidermal cells and stomata arranged along the longitudinal axis; (b), oblong cortical parenchyma cells; (c) and (d), fibers; (e) and (f), parenchyma cells; (g), bordered pitted vessels; (h), ring vessel and spiral vessel; (i), spiral vessel; (j), epidermis with glandular hair and non-glandular hair; (k), fragment of glandular hair. Major tissue fragments that appeared in the stem powder in (b) were longitudinal cells. Some parenchyma cells in (e) and (f) contained calciphytoliths and/or starch grains. Bar length: 50 µm. Fragments of powdered leaves of a cultivated \textit{N. tabacum} and commercial cigarettes (B): (a) and (b), epidermis consist of epidermal cells with sinuous anticlinal cell wall and stomata arranged anomocytic; (c), epidermal cells on the vascular bundle; (d) and (e), fragments of mesophyll containing chloroplasts in the powder of green leaf; (f), mesophyll with net-veins derived from cigarettes; (g), mesophyll with net-veins derived from brown leaf of cultivated plant; (h), fibers derived from cigarettes; (i) to (k), spiral vessel; (l), non-glandular hair; (m) and (n), glandular hair; (o) to (r), tissue fragments derived from petioles; (o), fragment of epidermis; (p), fragment of collenchyma; (q), long large vessels; (r), calciphytoliths contained in large parenchyma cells. Observed epidermal cells with sinuous anticlinal cell walls in (a) and (b) derived from leaf blades were the characteristic fragments of leaves. Some mesophyll parenchyma in (d) and (e) included calciphytoliths. Bar length: 50 µm. cd: calciphytoliths, pfs: simple perforation.

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