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Micromorphological and preliminary phytochemical studies of *Azadirachta indica* and *Melia azedarach*

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Abstract: *Azadirachta indica* A.Juss. and *Melia azedarach* L. are 2 closely related species from Meliaceae that are considered noteworthy medicinal plants in the world and have very often been confused with each other. Various evaluations like organoleptic and microscopic characteristics, preliminary phytochemical screening, and fluorescence analysis were carried out on the pulp, husk, leaves, and seeds of both plants in powdered form to establish appropriate data that can aid rapid and easy differentiation between them. The results showed that these 2 species can be differentiated macroscopically and microscopically but tests of phytochemical analysis were not absolutely felicitous for this aim. In addition, the obtained information about the different colours of the powders upon reaction with various reagents and fluorescence analyses of them provided supporting referential parameters for identification and differentiation between these 2 species.

Keywords: *Azadirachta*, fluorescence analysis, *Melia*, micromorphology, phytochemical screening

1. Introduction

Azadirachta indica A.Juss. (Meliaceae), popularly known as "neem", is a multipotential tree that originally belongs to South India and Myanmar and is cultivated on the southern coast of Iran. It is commercially used as a natural insecticide, pesticide, and agrochemical in various countries (Boeke et al., 2004; Koul & Wahab, 2004). Besides this, it is the one of the most versatile medicinal plants in that almost all of its parts (leaves, bark, flower, fruit, and root) have long been used in Iranian, Indian, and Chinese traditional medicine (Sharafkandi, 1991; Biswas et al., 2002; Khare, 2007) and have been scientifically shown to possess a variety of pharmacological properties, such as antiinflammatory, antipyretic, analgesic, immunostimulant, hypoglycaemic, and antiulcer properties. It also has pregnancy interceptive, antimalarial, antifungal, antibacterial, antiviral, anticarcinogenic, antihepatotoxic, and antioxidant properties (Ogbuewu et al., 2011; Zhou et al., 2011). *Melia azedarach* L., known as "Persian lilac" or "chinaberry", is another member of the Meliaceae family and is closely related to neem. This plant, which is abundantly distributed in the northern forests of Iran, is native to the Middle East (Ghahreman, 1887; Singh et al., 2009). Similarly, all parts of the Persian lilac are found to possess numerous medicinal properties, such as antioxidant (Ahmed et al., 2008; Nahak & Sahu, 2010), antihepatotoxic (Samudram et al., 2009), antiviral (Alche et al., 2002, 2003), antibacterial (Khan et al., 2008), antiparasitic (Szewczuk et al., 2003), antiulcer (Bahuguna et al., 2009), and pregnancy interceptive (Keshri et al., 2004) properties. It is a common herb used today in Iranian and Chinese traditional medicine (Sharafkandi, 1991; Zhou et al., 2011).

According to the literature, neem and Persian lilac have very often been confused with each other (Singh et al., 2009). However, they are morphologically different. Neem has pinnate leaves, 3-lobed stigmata, and 1(–2)-seeded drupes, whereas the Persian lilac has bipinnate leaves, 5-lobed stigmata, and up to 5-seeded drupes (Faridah Hanum & Van der Maesen, 1997). Wildcrafting and the sale of medicinal plants by uneducated people without enough knowledge may cause such unreliability in plant sources. The confusion is particularly more likely when the plant material is supplied in powdered form.

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Microscopic morphology is often a practical and reliable method for diagnosing the authenticity of powdered herbal material, especially when it is accompanied with phytochemical assays. The present study was carried out based on an investigation of the organoleptic and microscopic characteristics as well as a fluorescence and phytochemical analysis of neem and Persian lilac to define referential pharmacognostical and phytochemical parameters that can be used for the identification and differentiation of these 2 valuable species.

2. Materials and methods

2.1. Plant materials

The leaves and fruits of *Azadirachta indica* and *Melia azedarach* were collected from Gorgan (Golestan Province) and Bandar Abbas (Hormozgan Province), respectively, and their voucher specimens were deposited at the herbarium of the Faculty of Pharmacy of Tehran University of Medical Sciences.

The collected leaves and fruits were separately air-dried at room temperature. The dried fruits were dehusked and decorticated and their seed kernels were separated from their husk and pulp. The leaves, pulp and husk of the fruits, and seed kernels of each plant were separately crushed into fine powders and stored in closed containers for further use. Finally, 6 specimens were prepared and coded as follows: LN (leaves of neem), LP (leaves of Persian lilac), PN (pulp and husk of neem), PP (pulp and husk of Persian lilac), SN (seed kernels of neem), and SP (seed kernels of Persian lilac).

2.2. Organoleptic and microscopic study of powdered plant materials

All powdered specimens were evaluated for macroscopic appearance and organoleptic characters. In order to observe the microscopic structures of the powders, each of them was separately passed through a sieve (mesh no. 25), cleared by boiling with KOH (10%) for 0.5–3 min (depending on the stiffness of its tissue), and rinsed twice with sodium hypochloride and once with distilled water (Manayi et al., 2012). The slides were stained with specific stains such as methylene blue, carmine, or toluidine blue and observed under the microscope. Wherever necessary, different histochemical reagents, such as phloroglucinol (1%) and concentrated HCl (for lignified structures), H_2SO_4 (350 g/L, for calcium oxalate crystals), iodine solution (for starch granules), Sudan red G (for cuticular cell walls), and Sudan red G in acetic acid and ethanol (for essential oils, resins, fats, and fatty oils), were used on the bleached powders (Upton et al., 2011; WHO, 2011). All samples were observed under a Carl Zeiss Standard 14 Laboratory Microscope (Germany).

2.3. Preliminary phytochemical tests

Fifty grams of each powder was extracted using a percolation method that involved 3 consecutive extractions of 48 h with methanol/H_2O (80:20, v/v) at room temperature. Preliminary phytochemical tests of the extracts for alkaloids, phytosteroids, flavonoids, tannins, coumarins, saponins, and anthraquinone were carried out using specific reagents through prescribed standard methods (Sharifzadeh et al., 2006; Saeidnia, 2012).

2.4. Fluorescence analysis

The change in colour of powdered plant materials was studied separately in treatment with acids (H_2SO_4, picric acid, HNO_3, acetic acid, HCl), bases (NaOH in methanol, NaOH in water, NH_3), and other reagents ($AgNO_3$, $FeCl_3$, iodine, methanol). The fluorescence characters of the different powders with various chemical reagents were also observed under UV light (254 nm and 366 nm) (Kokoshi et al., 1958).

3. Results and discussion

Azadirachta indica and *Melia azedarach* are used to treat a broad range of ailments (Zargari, 1990). Despite differences in morphology and distribution areas, these closely allied species have long been erroneously confused with each other (Singh, 2004; Singh et al., 2009). Based on Aghili Khorasani (1844), this confusion was reported 2 centuries ago in Iranian traditional medicine. In some of the newer studies that have been conducted on these 2 species, the source of plant material is in doubt and sometimes it is impossible to find out which species has been studied (Puri, 1999; Rai & Carpinella, 2006). In that case, establishing suitable parameters is required for the differentiation of these species and their quality control. When the plant parts are supplied in their whole form, morphological differences may be useful for their identification. If, however, as is now common, crude plant materials are traded in powdered form, microscopic assessment at a cellular level or phytochemical analysis is needed to provide useful and reliable evidence. It is noteworthy that the importance of micromorphological characters is now well established for the identification of medicinal plant parts (Güvenç & Kendir, 2012; Albert & Sharma, 2013). In many cases, microscopic structures of closely allied species were completely different in contrast with their high similarities in morphological characters (Mostafavi et al., 2013).

Evaluation of the macroscopic appearance and organoleptic characteristics of the powders, as an initial

step, can be easily employed and contributes to their identification. The following are the results of this evaluation: LN and LP were both green with nearly identical aromatic odours. On the other hand, LN had an intense bitter taste distinct from LP, which tastes faintly bitter. PN and PP had slight bitter and fruity odours, respectively, while their tastes were both sweet at first and then became bitter. The odour of SN was fatty and rancid and its taste was intensely bitter, while SP was almost odourless and its taste was slightly bitter.

Many plant tissue elements, described as follows, were observed through the microscopic examination of the powdered samples: upper and lower epidermises of both LN and LP were covered by a thick cuticle. The

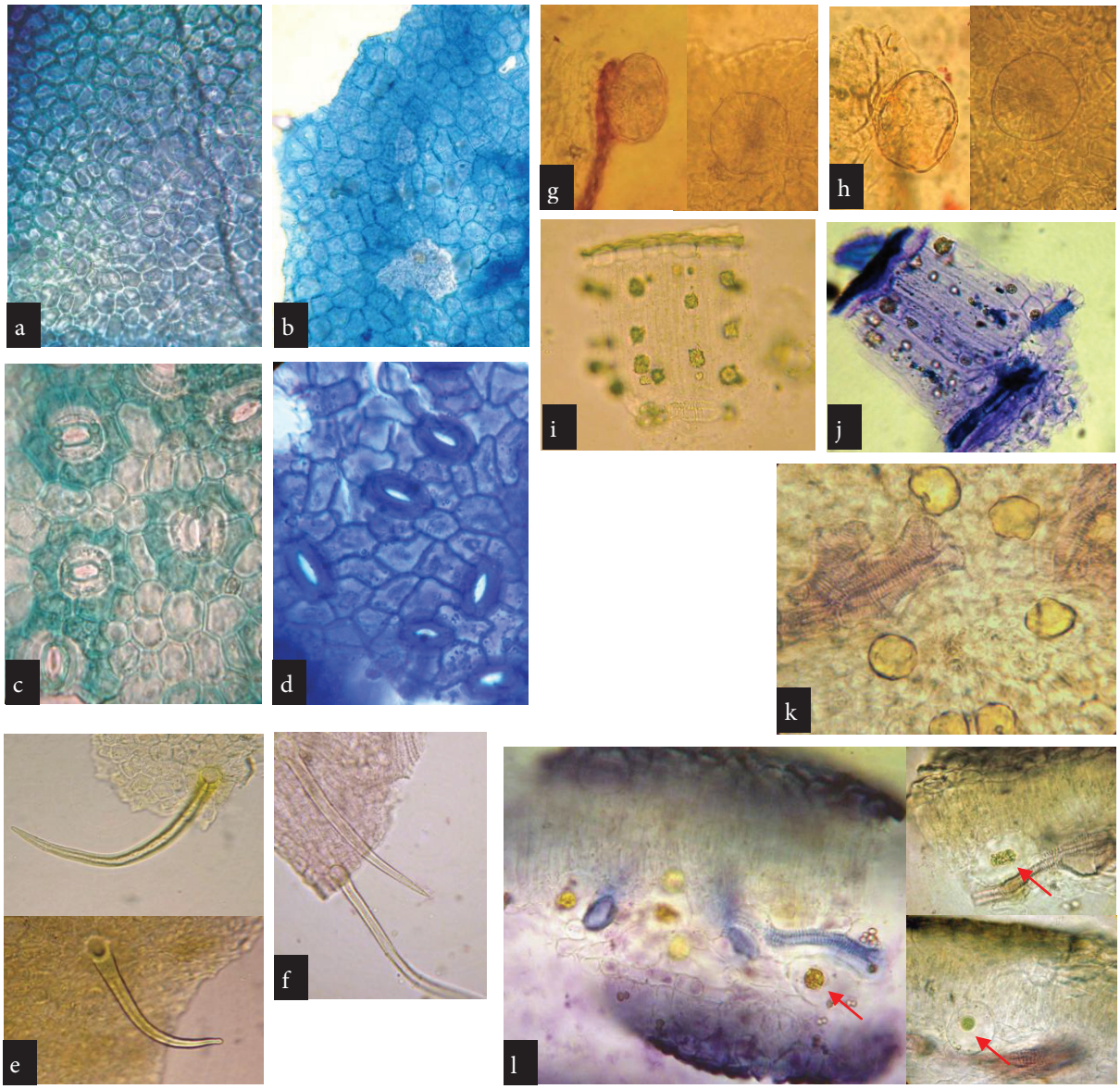

Figure 1. Comparison of the microscopic characteristics of powders of leaves. a- Upper epidermis of LN (40´). b- Upper epidermis of LP (40´). c- Lower epidermis of LN with actinocytic type of stomatal complexes (40´). d- Lower epidermis of LP with actino-stephanocytic type of stomatal complexes (40´). e- Unicellular covering trichomes of LN. f- Unicellular covering trichomes of LP. g- Peltate trichome in LN (lateral and surface view; 40´). h- Peltate trichome in LP (lateral and surface view; 40´). i- Idioblasts containing cluster crystals of calcium oxalate in LN (40´). j- Idioblasts containing cluster crystals of calcium oxalate in LP (40´). k- Yellow secretory cells and veins (surface view; 100´). l- Yellow secretory cells, shown by arrows, situated near veins (transverse view; 40´).

upper epidermis of both leaves consisted of polygonal cells without stomata (Figure 1), whereas stomata were abundant in the lower epidermises (Figure 1). The walls of upper epidermal cells in LN were thicker than those in LP. In addition, the walls of the lower epidermal cell in LP were more angled and wavier in contrast to those in NP, which were more curved and straight. The stomatal complexes of LN were predominately of the actinocytic type, but the majority of the stomatal complexes in LP seemed to be intermediate between actinocytic and stephanocytic (actino-stephanocytic type). This difference might be due to the more waviness of subsidiary cell walls in LP, which disarranges their radiated pattern. The guard cells of LN and LP were often surrounded by 6 and 6–8 subsidiary cells, respectively. Unicellular covering trichomes were found in both plants, mostly on the upper epidermis (Figure 1). They were tapering and pointed, and most of them were bent at an obtuse angle near the middle. However, some of them were straight or curved. Peltate trichomes were similarly found in both plant leaves (Figure 1). These trichomes had a short stalk and a round head and, in contrast to the covering trichomes, their occurrence was very infrequent. The leaves of *Aglaia*, from Meliaceae, frequently possess this type of trichome. They also rarely occur in another genus of Meliaceae, *Trichilia* (Muellner et al., 2005). Idioblasts containing cluster crystals of calcium oxalate were scattered between palisade parenchyma in both LN and LP (Figure 1). However, they were more obvious in LP. LN also contained abundant yellow secretory cells, which were embedded between spongy cells near veins (Figure 1). As these elements were absent in LP, they can be very useful in differentiating the 2 plant leaves microscopically. The contents of the mentioned secretory cells were stained orange-red with Sudan red G in acetic acid and then dissolved in ethanol. According to the WHO (2011), these cells contain essential oils or resins. Fragments of lignified vessels with spiral thickening were found in the leaves of both plants, but they may not provide significant characters to identify them.

The powder microscopy of PN revealed the presence of small, polygonal, squarish, or rectangular cells in exocarp (Figure 2). The cells were arranged in groups of 2–4 cells, which were surrounded by thick walls, while within the groups, the walls were much thinner. The exocarp in PN also contained thick-walled, lignified brachysclereids occurring singly or in groups (Figure 2). Exocarp in PP just

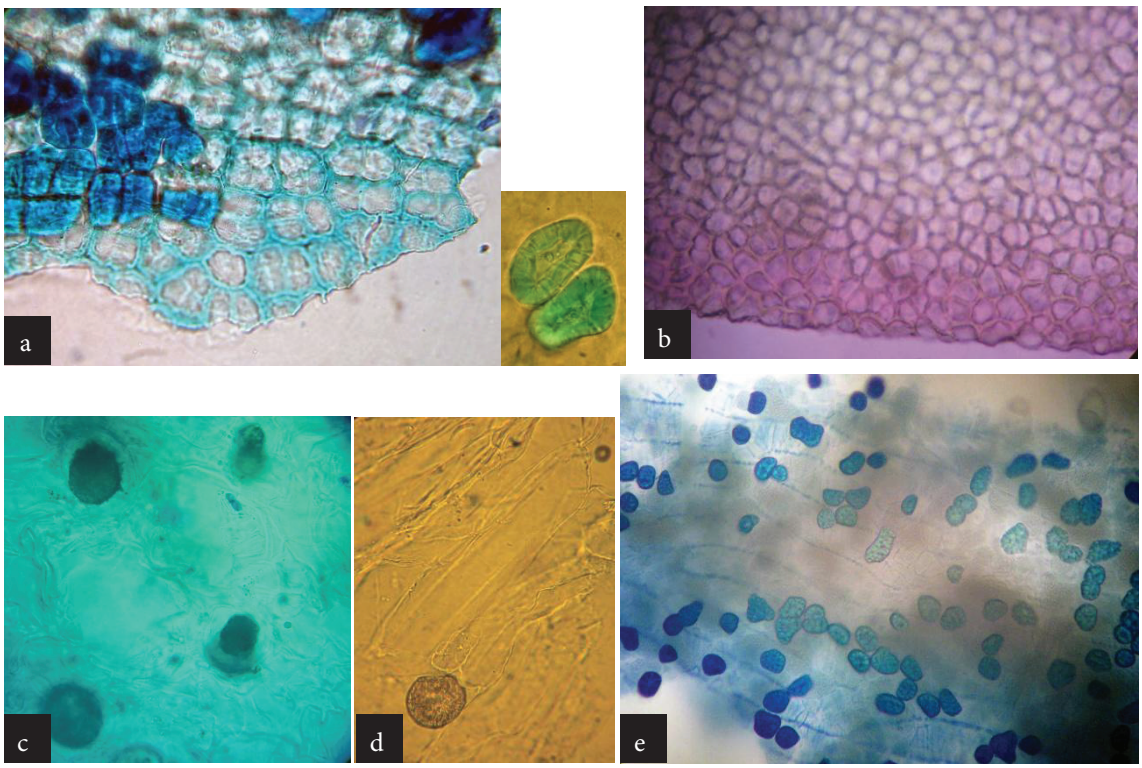

Figure 2. Comparison of microscopic characteristics of powders of fruits. a- Exocarp of PN with lignified brachysclereids (left picture: 40´; right picture: 100´). b- Exocarp of PP (40´). c- Mesocarp in PN (40´). d- Mesocarp in PP (40´). e- Testa epidermis of SP with small brachysclereids (40´).

consisted of simple small polygonal cells (Figure 2). The mesocarps of both fruits comprised large, colourless thin-walled parenchymatous cells and embedded differently sized secretory cells filled with yellowish brown contents (Figure 2). Testa epidermis of SP showed elongated bordered-pitted cells with scattered small brachysclereids (Figure 2). This structure was observed abundantly in prepared slides of SP powder in contrast to the testa epidermis of SN, in which it was not detected in any slide of this sample. This is due to the anatomical differences of these 2 seeds. The seed of the Persian lilac is smaller and possesses thick stiff testa, while the seed of neem is much bigger with considerably thinner and somewhat indistinct testa occupying a small part of the whole seed. Cotyledons of both plant seeds are composed of parenchymatous cells containing abundant oil droplets.

The obtained results from the preliminary phytochemical tests were mostly similar for the 2 species. Alkaloids, flavonoids, phytosterols, and coumarins were detected in all extracts, but anthraquinone was not found. Furthermore, all specimens contained tannins except SN (Table 1). Only the presence of saponins in various extracts was differentiating. Ayoola et al. (2008) reported similar results for the stem bark of *A. indica*, such as the presence of terpenoids, flavonoids, saponins, tannins, and absence of anthraquinones. However, alkaloids were not detected in the mentioned part of the plant.

The results of the florescence assay on the plant powders with 13 different reagents were separately observed under daylight and UV radiation (Table 2). According to Table 2, the behaviours of the 2 seed kernel powders in reaction with the different reagents are more diagnostic than the pulp or leaf samples, yet this assay can definitely differentiate these 2 plant powders from each other.

The obtained results from the phytochemical tests showed that the chemical profiles of *A. indica* and *M. azedarach* are very similar. The similar compounds isolated from these 2 species may support this idea (Rai & Carpinella, 2006). In that case, authentication and differentiation of these plants by phytochemical methods seems to be difficult while dissimilar micromorphological structures are readily distinguishable. Based on the present study, it can be deduced that micromorphological study is a practical and affordable method for differentiating these 2 species, although to obtain this benefit, the fluorescence analysis can be considered as its great complement.

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Table 1. Preliminary phytochemical tests of the pulps, leaves, and seeds of *Melia azedarach* and *Azadirachta indica*.

		Extract	LN	LP	PN	PP	PN	SP
Phytochemicals	Test name	Reagent*						
Alkaloids	Wagner	Potassium mercuric iodide	+	+	+	+	+	+
	Mayer	Iodine in potassium iodide	+	+	+	+	+	+
Flavonoids	Cyanidin test	HCl (37%) + Mg powder + amyl alcohol (50%)	+	+	+	+	+	+
Phytosterols	Libermann–Burchard test	Acetic acid + conc. H_2SO_4	+	+	+	+	+	+
	Salkowski test	Conc. H_2SO_4	+	+	+	+	+	+
Tannins		FeCl3	+	+	+	+	+	+
	Gelatin test	NaCl (10%) + gelatin (1%)	+	+	+	+	+	+
Coumarins	TLC	Ethanolic KOH	+	+	+	+	+	+
Saponins	Foam test	–	+	–	+	–	–	+
Anthraquinones	Borntrager test	NH_3	–	–	–	–	–	–

*Prepared based on words of Wagner and Bladt (1996) and Saeidnia (2012).

Table 2. Fluorescence analysis of leaf, pulp, and seed powders of *Azadirachta indica* and *Melia azedarach* under UV radiation.

Treatment \ Sample name		PN	PP	LN	LP	SN	SP
As such	1	L brown	L brown	Green	Green	Milky	L brown
	2	Bluish white	Dirty yellow	Charcoal grey	Charcoal grey	Bluish white	S white
	3	Bluish white	Brownish black	S charcoal grey	S charcoal grey	Charcoal grey	Charcoal grey
H_2SO_4 conc.	1	Burnt brown	Reddish brown	D purplish brown	Purplish brown	Reddish violet	Purplish brown
	2	Black	Black	Black	Black	Violet	Violet
	3	Black	Black	Black	Black	D purplish brown	D purplish brown
NaOH (1 N) in methanol	1	L brown	L brown	Yellowish green	Yellowish green	Milky	L goldenrod
	2	Blue	Grey	Purplish blue	Blue	Green	Blue
	3	Amber	Khaki	Yellowish green	Yellowish green	Yellow	L goldenrod
Picric acid (saturated)	1	Amber	Amber	Phosphoric yellow	Phosphoric yellow	Yellow	L goldenrod
	2	D brown	D brown	D brown	D brown	Black	Black
	3	Amber	Amber	Greenish yellow	Greenish yellow	Greenish yellow	Greenish yellow
NaOH (1 N) in water	1	D orange	L brown	Yellowish green	D olive green	Milky	L brown
	2	Olive green	D green	Yellow	Yellow	Yellow	Blue
	3	D orange	L brown	Greenish yellow	Olive green	Green	Brown
HNO_3 50%	1	Yellow	Orange	Orange	Orange	Brown	Orange
	2	Black	Black	D brown	D brown	D green	D green
	3	Yellow	D orange	Orange	Orange	Yellow	Yellow
Methanol	1	L goldenrod	L brown	L green	Green	Milky	L goldenrod
	2	Blue	D green	Blue	Blue	Blue	Milky
	3	Grey	Greenish brown	D green	D green	Grey	Brown
$AgNO_3$ (1 N)	1	L brown	D violet	D green	D violet	Violet	Brown
	2	Orange	D violet	D brown	Black	Brown	Brown
	3	Bluish green	Brown	D violet	D violet	Brown	Brown
HCl conc.	1	D chocolate brown	Khaki	Brownish green	Brownish green	L goldenrod	D brown
	2	D brown	D brown	D S green	D S green	L goldenrod	D brown
	3	Burnt brown	Burnt brown	D green	D green	Brown	Brown
NH_3	1	L goldenrod	D goldenrod	D green	D green	White	Brown
	2	Green	Green	D green	D green	Green	Green
	3	L goldenrod	L brown	D green	D green	White	White
Acetic acid (glacial)	1	L goldenrod	L brown	Olive green	Yellowish green	Milky	L brown
	2	Greenish yellow	Greenish yellow	D bluish green	Blue	Greenish yellow	Greenish yellow
	3	Greenish white	Brown	D green	D green	White	D goldenrod
$FeCl_3$ 5%	1	Khaki	Brown	Olive green	Olive green	Burnt brown	Brown
	2	WO	WO	WO	WO	WO	WO
	3	WO	WO	WO	WO	WO	WO
Iodine	1	Black	D reddish brown	Reddish brown	Reddish brown	D green	Reddish brown
	2	WO	WO	WO	WO	WO	WO
	3	Brown	Brown	Brown	Reddish brown	Brown	Reddish brown
HCl (1 N)	1	Black	Khaki	D green	D green	Violet	Black
	2	S black	S black	Bluish green	Bluish green	Bluish green	Brown
	3	Black	Black	Brown	Brown	Grey	Brown

Abbreviations: 1: visible; 2: 366 UV; 3: 254 UV; L: light; S: shiny; D: dark; WO: without distinct fluorescence.

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