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Full Length Research Paper

Structural organization and phytochemical analysis of *Pimenta dioica* (L.) Merrill (Myrtaceae) leaves collected from Goiás State, Brazil

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Several biological properties are attributed to the *Pimenta dioica* plants that are native to Central America. Given the possible variabilities in both the morpho-anatomical and chemical aspects of the species that grow in different locations, this study aimed to contribute to the pharmacognostic study of *P. dioica* leaves from Brazil. Therefore, we morpho-anatomically described the plants, performed a phytochemical screening, analyzed the chemical composition of the essential oil and determined the quality parameters of *P. dioica* leaves that were collected in Goiás State. The leaves are simple and exhibit a camptodromous-brochidodromous venation pattern. The blades exhibit anomocytic stomata on the abaxial side, a dorsiventral mesophyll, secretory cavities on both sides, and a uniseriate epidermis. Trichomes can be observed in the petiole. The tests revealed the presence of phytochemical saponins, tannins and flavonoids in the samples. The major components of the essential oil were eugenol (60.8%) and myrcene (19.3%). The results regarding the moisture content (7.87% w/w), total ash (8.84% w/w), HCl-insoluble ash (1.74% w/w) and total flavonoids (1.88% w/w) can be used as parameters for quality control of botanical material. Additionally, the results of this study indicates strong similarities between the chemical compositions of the leaves of *P. dioica* plants that were grown in Goiás, Brazil and those from other parts of Brazil and the world.

Key words: Myrtaceae, essential oils, eugenol, morpho-anatomy, *Pimenta dioica*.

INTRODUCTION

The Myrtaceae family is one of the most important plant families worldwide, contains approximately 130 genera and 4000 species, has pantropical and subtropical distributions, and contains species of great medical

interest (Landrum and Kawasaki, 1997; Judd et al., 1999; Joly, 2002; Souza and Lorenzi, 2005). Among this family is the genus *Pimenta*, which contains 14 species that are native to Central America and a single species that is

native to Brazil, *Pimenta pseudocaryophyllus* (Gomes) LR Landrum (Landrum and Kawasaki, 1997).

Among the species that are native to Central America, *Pimenta dioica* (L.) Merrill (Figure 1a and b) was introduced to Brazil, where it can be found as an ornamental tree (Lorenzi et al., 2003). Popularly known as "pimenta-da-jamaica", "malaqueta", and "pimento", *P. dioica* is an evergreen tree that can reach 7 to 10 m in height and is native to Mexico, Guatemala, Belize, Honduras, Nicaragua, El Salvador, Cuba and Jamaica (Landrum, 1986; Lorenzi et al., 2003). The fruits of this species are used in confectionery because they express an aroma and flavor similar to the combination of clove, cinnamon and bay. For this reason, the fruits are known as "allspice" (Lorenzi et al., 2003). In addition to the use of *P. dioica* as a condiment or spice, the people of Central America and the Caribbean use preparations made from *P. dioica* leaves for the treatment of hypertension, diabetes, obesity, digestive disorders, dysmenorrhea and abdominal pain (Zhang and Lokeshwar, 2012). Scientific studies conducted using crude extracts, fractions or essential oils from the leaves of specimens from Central America have uncovered several biological characteristics, including antinociceptive, antipyretic, antidermatophyte, antimicrobial, hypotensive, central nervous system depressant, anti-hemorrhagic, anticancer and antioxidant activities (Benítez et al., 1998; Oussalah et al., 2006, 2007; Marzouk et al., 2007; Khandelwal et al., 2012; Zhang and Lokeshwar, 2012; Shamaladevi et al., 2013).

The scientific literature has shown that the essential oils from the leaves have contributed to many of the biological activities of *P. dioica*; the eugenol of *P. dioica* is a major component that has frequently been identified (Hernández et al., 2003; Oussalah et al., 2006, 2007; Zhang and Lokeshwar, 2012). In addition to these substances, some authors have identified flavonoids and tannins in the leaves of *P. dioica* (Castro et al., 1999; Marzouk et al., 2007; Nitta et al., 2009). A recent search of scientific databases revealed a lack of studies of Brazilian specimens of this important medicinal species. As demonstrated earlier, research has evaluated specimens that are native to Central America. Variability, both morpho-anatomical and chemical, between the species from different locations, is possible due to adaptive responses (Dickison, 2000; Simões and Spitzer, 2010; Cunha et al., 2005) that might have resulted in distinct or even unique biological activities of the exotic species.

According to Jorge et al. (2005), research and

microscopic analyses are essential for the identification of plant products and allow for the identification of fraud and possible adulterations. The data from anatomical analyses have unique values in taxonomic studies as stated by Johnson (1980) and Van Wik et al. (1982). The existence of morphological and anatomical references is extremely important, particularly for plants that are used in popular medicine, so that the samples can be confirmed and to enable tests of authenticity that are needed because confusion between morphologically similar species which are very common and can lead to the improper use of a particular plant. Thus, the anatomical characteristics add to the set of information that allows for the correct identification of plant species.

This study aimed to conduct a pharmacognostic analysis of the leaves of *P. dioica* from Brazil. This analysis included a description of the petiole and leaf blade structures, a phytochemical screening, an analysis of the chemical composition of the essential oil using gas chromatography coupled to mass spectrometry (GC-MS) and the determination of parameters that can be used for identification and plant material quality control.

MATERIALS AND METHODS

Plant

The plant material was collected from *P. dioica* specimens that were cultivated as ornamental trees in the cities of Goiânia, Goiás State (16° 40' 21.6" S and 49° 14' 30.9" W, 731 m) in October, 2007 and inhumas, Goiás State (16° 35' 33.9" S and 49° 49' 39.8" W, 780 m) in September, 2011 on public roads. The plants were identified by Professor Dr. José Realino de Paula of the Universidade Federal de Goiás, and the voucher specimens were deposited, respectively at the Herbarium of the Universidade Federal de Goiás (UFG-40.112) and at the Herbarium of the Universidade Estadual de Goiás (HUEG-6779). The leaf samples were air-dried in a chamber at 40°C, ground into a powder, packaged, labeled and stored until use in the phytochemical assays. The morpho-anatomical study was performed using fresh adult, fully expanded leaves that were collected below the third node.

Morpho-anatomical study

The macroscopic leaf characterization was performed by observation with the naked eye and a stereoscopic microscope when necessary according to the parameters that were described by Oliveira et al. (1998) and Oliveira and Akisue (2003). The leaf venation pattern was determined based on the parameters described by Cardoso and Sajo (2006) after leaf diaphanization. The fresh material was diaphanized according to the method of Kraus and Arduin (1997) and visualized using a stereomicroscope

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(Leica model EZ4D). Using an average of 5 leaves, sections of approximately 1.0 × 0.5 cm of fresh leaf were excised by hand from the following regions of the leaf blade: the midrib segments, the internodal regions and the edges. In the middle region of the petiole sections, sections of approximately 0.5 cm were excised. These sections were fixed in 37% formaldehyde, propionic acid and 70% ethanol (FPA) at a ratio of 1:1:18 (V/V) for 3 days (Kraus and Arduin, 1997) and then stored in 70% ethanol. To mount the slides, transverse sections were cut by hand, clarified with 30% sodium hypochlorite (V/V), washed with distilled water, neutralized with 5% acetic acid, washed again with distilled water, double-stained with Alcian Blue:Safranin (9:1) and mounted onto slides with 50% glycerol (V/V) according to a technique adapted from Bukatsch (Kraus and Arduin, 1997). The fresh paradermal leaf sections were treated as described. Approximately, three slides, each containing three sections, were prepared from each leaf region and mounted in a colorless varnish. Photomicrographs of the anatomical structures were obtained using a photomicroscope (ZEISS-Axioskop) with the photographic film Kodacolor ASA 100. The illustration scales were obtained under the same optical conditions. Scanning electron microscopy (SEM) was performed at the LabMic of the Universidade Federal de Goiás. The fresh leaf sections were fixed in Karnovisk (Kraus and Arduin, 1997), dehydrated in an ethanol series and subjected to a CO₂ critical-point evaporator (Autosamdri). The specimens were mounted on aluminum ("stubs") and metalized with gold (Denton vacuum). The leaves were examined in a frontal and transverse scanning electronic microscope (Jeol, JSM – 6610, equipped with EDS, Thermo scientific NSS Spectral Imaging).

Determination of the plant material quality parameters

The tests for determining the humidity, total ash and HCl-insoluble ash, which were used as the plant material quality parameters, were performed in triplicate according to the Brazilian Pharmacopoeia V (Brazil, 2010). The volumetric method (Karl Fisher) was used to quantify the humidity content. The results are expressed as the mean of three replicates, and the standard deviations were calculated.

Phytochemical screening

The qualitative analysis of the major classes of secondary metabolites was performed according to methods that were adapted from Costa (2001), Matos (2009) and Matos and Matos (1989). Tests were performed to detect the presence of anthraquinone heterosides, digitalis heterosides, flavonoids, saponins, tannins, alkaloids and coumarins. The total flavonoid content was determined by the method described in the calendula monograph as stated by the Brazilian Pharmacopoeia V (Brazil, 2010) in triplicate, and the results are expressed as the means and the standard deviations.

Analysis of the essential oil

The leaf samples were ground into a powder and submitted to hydrodistillation in a modified Clevenger-type apparatus (2 h). The essential oils were dried over anhydrous Na₂SO₄, the yields were measured, and the oils were stored at -18°C for further analysis. The GC-MS analysis was performed at the Institute of Chemistry of the Universidade Federal de Goiás on a Shimadzu QP5050A instrument. The column, a CBP-5 (Shimadzu)-fused silica capillary

column (30 m long × 0.25 mm i.d. × 0.25 µm film thickness, composed of 5% phenyl methylpolysiloxane), was connected to a quadrupole detector operating in EI mode at 70 eV. Helium was used as the carrier gas at a flow of 1 ml min⁻¹. The injector and interface temperatures were 220 and 240°C, respectively, and the split ratio was 1:5. The injection volume was 0.5 µl (10% in hexane), and the oven temperature program consisted a ramp from 60 to 240°C at 3°C min⁻¹ followed by an increase to 280°C at 10°C min⁻¹, and ending with 5 min at 280°C. The analysis was conducted in the scan mode with a mass range of 40 to 400 m/z. The quantitative result was obtained by integrating the total ion chromatogram (TIC). The essential oil constituents were identified by comparing their mass spectra to those from the National Institute of Standards and Technology (NIST, 1998) and by comparing the mass spectra and the calculated linear retention indices (RI) with the corresponding values in the literature (Adams, 2007). The retention indices were obtained by co-injection with a C8-C32 mixture of linear hydrocarbons (Sigma, USA) and by the equation of Van den Dool and Kratz (1963).

RESULTS AND DISCUSSION

Morpho-anatomical study

The *P. dioica* leaves are simple, oblong-elliptic or elliptic-lanceolate and shortly petiolate (Figure 1c). The leaf blade is intact and varies from coriaceous to subcoriaceous. The blade measures 3.3 to 7.3 cm × 2.6 to 5.3 cm. The apex is obtuse to slightly emarginate. The leaf base is a symmetrical cuneata. The leaves are glabrous and overwrought on the adaxial surface with a glossy dark green coloration. There are no trichomes on either the adaxial or abaxial surfaces. The leaves exhibit a pinnate venation with the primary and secondary veins prominent on the abaxial surface, and the adaxial surface is printed on. The venation pattern (Figure 1d) is a pinned type camptodromus-brochidodromus in which the ribs diverge from the midrib at different angles and bend and arc (Figure 2a and b) before reaching the leaf margin. A similar venation pattern have also been recorded by Donato and Morretes (2009) for *Eugenia florida* DC, by Paula et al. (2008) for *P. pseudocaryophyllus* (Gomes) LR Landrum and by Cardoso and Sajo (2006) for other species of Myrtaceae. The petiole is curved and varies from circular to biconvex, measures 0.5 to 0.7 cm in length, and its insertion and surface are marginal and striated, respectively.

The morphological characteristics of the leaves that were examined in this work are in accordance with those described by Joly (2002) and Judd et al. (1999) for Myrtaceae. The characteristics also accord with the morphology described by Landrum (1986) and are highly similar to the descriptions of Donato and Morretes (2005) for *Psidium widgrenianum* Berg., Paula et al. (2008) and Farias et al. (2009) for *P. pseudocaryophyllus* and Donato and Morretes (2011) for *Myrcia multiflora* (Lam.) DC.

From the front view, the adaxial epidermal cells have

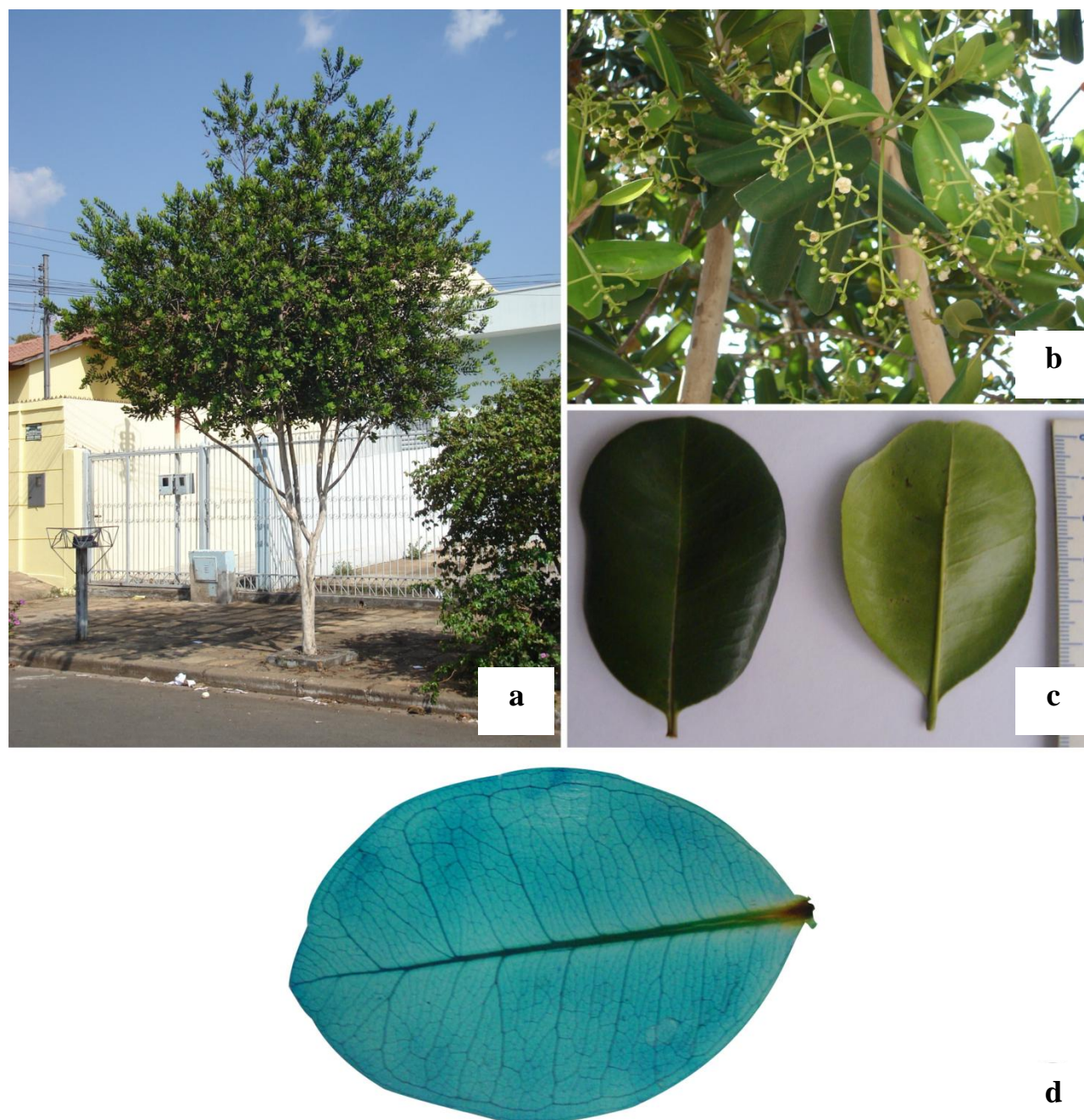


Figure 1. *Pimenta dioica* (L.) Merrill (Myrtaceae). (a) The general aspects of the plant; (b) the general aspects of the inflorescence; (c) the general aspects of the adaxial and abaxial surfaces of the leaves; (d) stereomicroscopy after clearing the leaf surface showing the details of the venation pattern.

irregular shapes and sizes, thick anticlinal walls that are predominantly punctuated, straight to slightly sinuous and lack stomata (Figure 2c). Secretory cavities are present in large numbers and are covered by pair of reniform cells that are surrounded by approximately 10 cells that are

smaller than the other epidermal cells (Figure 2c and d). Using scanning electronic microscopy (Figure 2d), it was possible to observe the corresponding holes in the tops of the secretory cavities, but the contours could not be observed due to a thick cuticle layer covering the entire

epidermal surface that assumed aspects in the granular cuticle along the entire length and exhibited some flat regions. According to Vannucci and Rezende (2003), the cuticle is formed by cutin (a lipid complex substance) and cuticular wax, and various strata or layers are formed by cutin and cuticular cellulose. Variable thicknesses can result in different patterns of ornamentation that are thicker in plants in dry conditions that receive sunlight radiation and have an important function in reducing plant water loss (Cutler et al., 2011).

From the front view, the cells of the abaxial epidermis (Figure 2e) also have irregular shapes and sizes and anticlinal punctuated walls, but these walls are thinner than those of the adaxial epidermis. The anticlinal walls are straight or slightly winding. There are many anomocytic stomatas surrounded by 3 to 5 epidermal cells (Figures 2e to 3a). Notably, secretory cavities are rarely present (Figures 2e and f). The pairs of kidney-shaped cells that overlap the secretory cavities are surrounded by approximately 7 smaller cells that have a characteristic shape and size that differs from those of the epidermal cells. The contours of the epidermal cells are thin (Figures 2f and 3a) due to the thick layers of the cuticle coats, but it was possible to observe numerous stomata and some secretory cavities. The cuticle on the surface exhibits aspects that range from smooth to rough, and some sparse protrusions are present (Figures 2f and 3a). In both epidermises, the cells have thick anticlinal walls that are similar to those described by Paula et al. (2008) for *P. pseudocaryophyllus*. According to Gomes and Neves (1993/1997), the outlines of the anticlinal walls of the epidermal cells can display variations within the Myrtaceae family.

In transverse sections, the epidermis is unistratified and typically has a dorsiventral mesophyll (Figures 3b to d). The epidermal cells of the adaxial surface are larger than those of the abaxial surface. The abaxial epidermal cells are aligned such that they are more compact than the adaxial epidermal cells (Figures 3b to d). Both epidermises are coated on their external anticlinal walls with a cuticle layer, and the adaxial surface cuticle seems to be thicker than the abaxial surface and extends toward the anticlinal walls to form cuticular flanges (Figure 3e). The mesophyll consists of 2 to 3 layers of palisade parenchyma. The first layer is formed by cells that are very compactly arranged in a rectangular palisade, and the second layer has smaller cells that are followed by spongy parenchyma (Figures 3b to d). The spongy parenchyma is formed by cells that are loosely distributed in approximately 8 strata in which the conductive fabric that can extend the palisade parenchyma is located (Figure 3b). In the sub-epidermal region at the time of the observations of the vascular bundle, 2 to 4 layers of angular collenchyma were observed on both sides. The rounded parenchymal cells were of varying dimensions

and surrounded the vascular bundle side, which was surrounded by sclerenchymal fibers.

The schizogenous secretory cavities are spherical and internally bound by flattened cells that are found more frequently below the adaxial epidermis (Figures 3b, c, e and 4). The lumen of the secretory cavity communicates with the outside through 4 to 5 pairs of cells that are immersed in the epidermis and palisade (Figures 3e and 4a). Although in the mesophyll it is possible to observe a large number of idioblasts, carriers of calcium oxalate crystals in the form of drusen can also be observed (Figure 4b). In the leaf margin (Figure 4c), the mesophyll cells are gradually replaced by the angular collenchyma to end in approximately 5 layers of sub-epidermal cells. There, the secretory cavities and epidermal cells are covered by a thick cuticle layer. This presence of this cuticle travelling to the leaf margin has also been noted in the studies of Gomes and Neves (1993/1997).

In transverse sections, along the main vein (Figure 4d) from the periphery to the center, a thick cuticular layer of epidermis covering the adaxial and abaxial surfaces is present, and it is unistratified. Cuticular flange can also be observed. In the sub-epidermal region on both sides but with greater frequency on the abaxial surface, secretory cavities and a variable number of layers of lacunar collenchyma can be observed. The parenchyma cells surround the vascular bundle core. The cortical parenchyma is formed by round cells that often contain idioblasts with calcium oxalate crystals in the form of druse. Similar observations have been made in the genus *Gomidesia* (Myrtaceae) by Gomes and Neves (1993/1997) regarding the provision of crystals throughout the parenchyma and the presence of secretory cavities on both sides.

The central vascular bundle is arranged in the form of a continuous bicollateral arc in which the xylem appears slightly compressed and is surrounded by the phloem internally and externally; this characteristic was also described by Paula et al. (2008) for *P. pseudocaryophyllus* and Siqueira-Nunes and Martins (2010) for *Syzygium cumini*. Near the adaxial surface, the vascular system is thickly surrounded by a sclerenchymatic sheath. There are still a number of crystals scattered throughout the parenchyma and phloem that might, in some regions, constitute a crystal series (the most common condition in the phloem). In cross-section, the petiole presents itself as a circular, biconvex (Figure 4e), slightly thick cuticle coupled with a layer of epidermal cells. There are some trichomes. The vascular system is arranged in a bicollateral arc. The cortex is collenchymatous and filled with petiole parenchymal cells with thick walls. There are also numerous idioblasts that contain calcium oxalate crystals in the form of drusen. In the subepidermal region, secretory cavities were present as in the leaf blade.

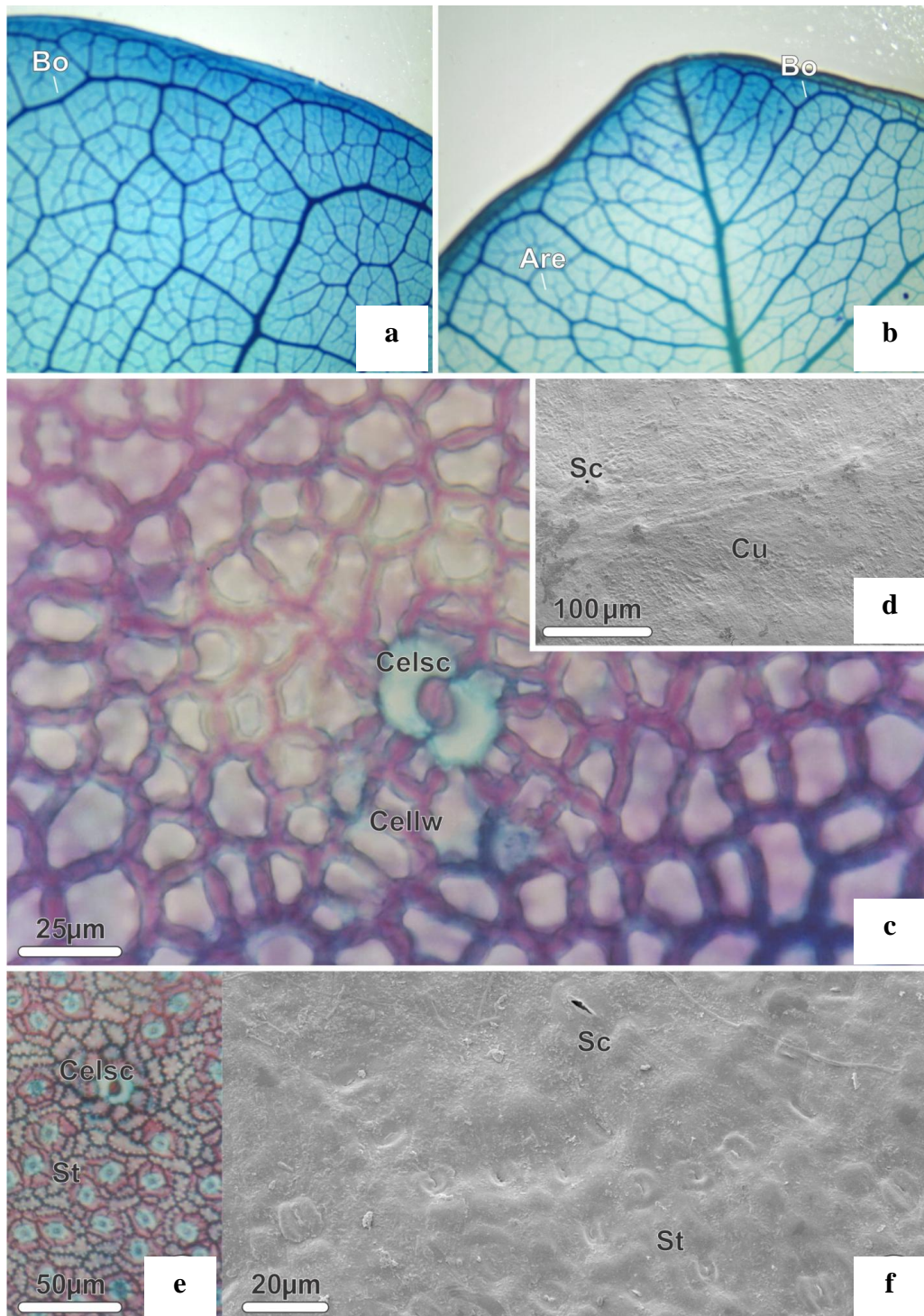


Figure 2. *Pimenta dioica* (L.) Merrill. (a and b) Stereoscopic microscopy showing details of the leaf venation pattern; (c) photomicrograph from the front view showing the adaxial epidermal cells that cover the secretory cavity; (d) electron micrograph from the front view showing the adaxial epidermis, the anomocytic stomata and the cell wall; (e) photomicrograph from the front view showing the abaxial epidermis, the anomocytic stomata and the cell wall; (f) front-view electron micrograph of the abaxial epidermis. Bo – bows, Are – areolas, Celsc– cells that cover the secretory cavity, Sc – secretory cavity, Cu – cuticle, St – stomata, Cellw – cell wall.

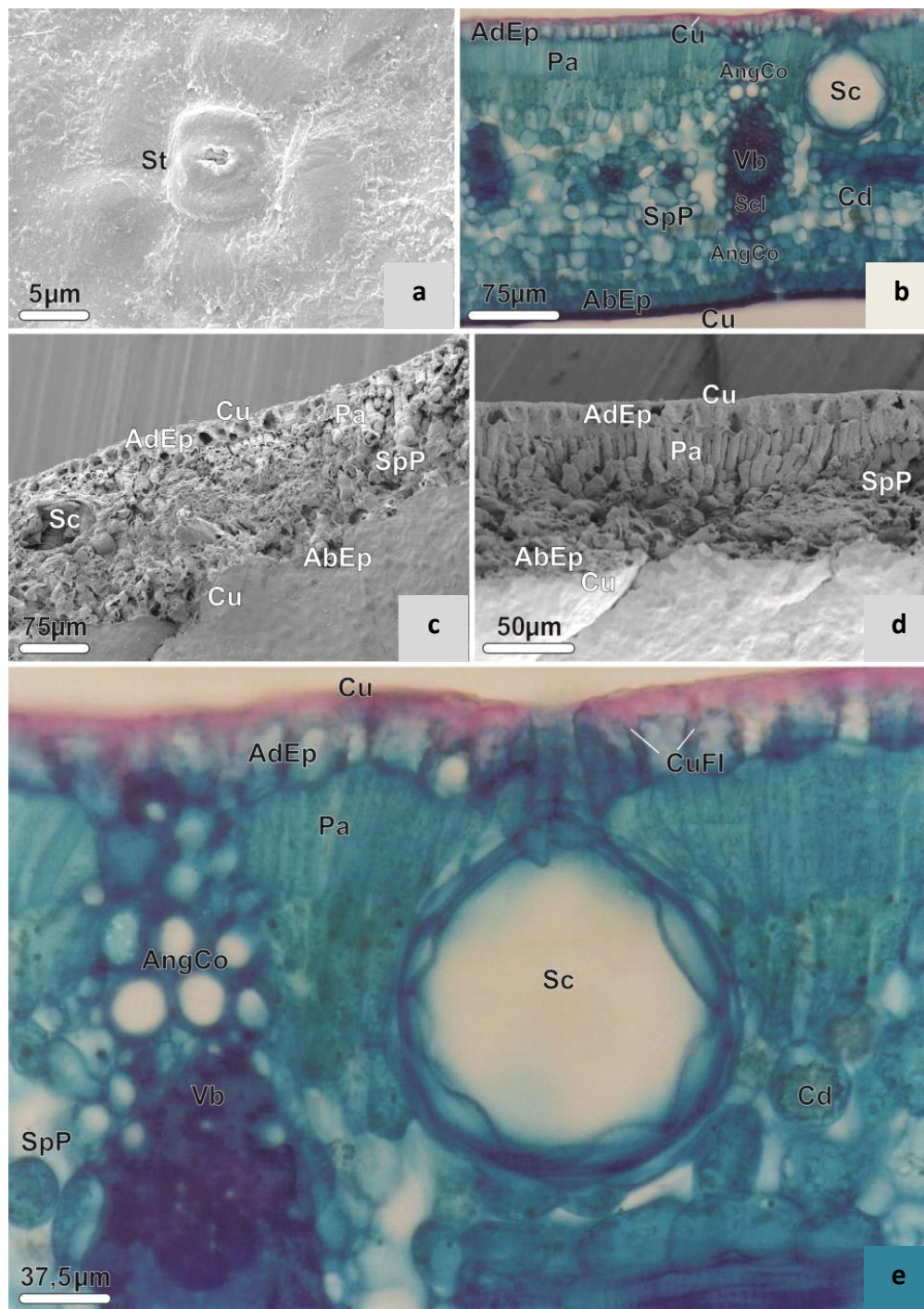


Figure 3. Leaf blade of *Pimenta doica* (L.) Merrill. (a) Electron micrograph from the front view showing details of the anisocytic stomata; (b) photomicrograph of a cross section showing the internal region; (c) electron micrograph of a cross section showing the mesophyll; (d) electron micrograph of a cross section showing the organization of the parenchyma along the leaf lamina; (e) photomicrograph of a cross section showing details of the secretory cavity cells, cuticular flanges on the walls of the adaxial epidermal cells and collenchyma. Cd – crystal (druze), AngCo – angular collenchyma, Sc – secretory cavity, Cu – cuticle, AbEp – abaxial epidermis, AdEp – adaxial epidermis, S – stomata, Scl – sclerenchyma, CuFl – cuticular flange, Vb – vascular bundle, SpP – spongy parenchyma, Pa – palisade.

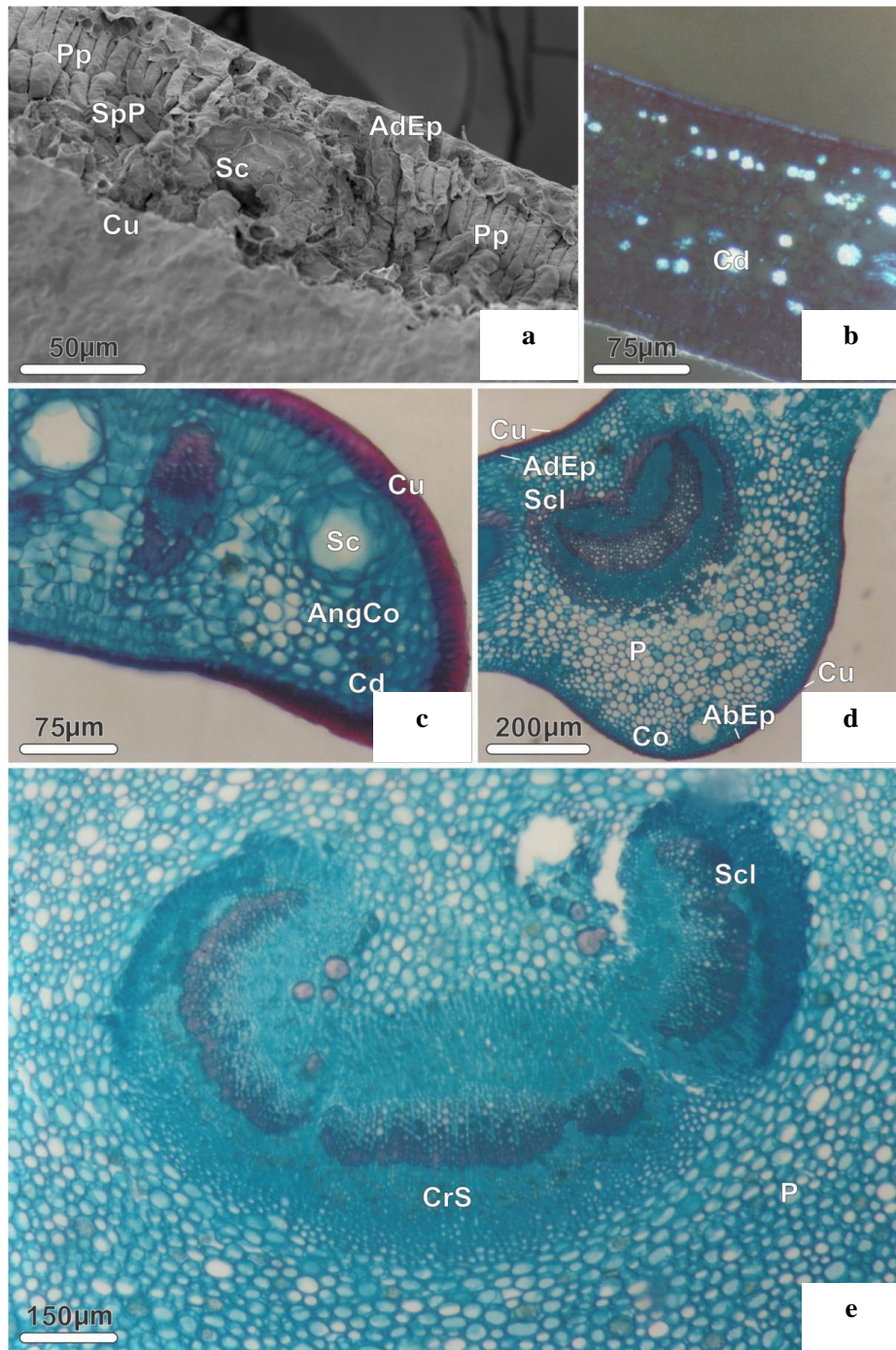


Figure 4. Leaf blade of *Pimenta dioica* (L.) Merrill. (a) Electron micrograph of a cross section showing the internal cavity lining and secretory cells that form the lumen; (b) photomicrograph of a cross section under polarized light showing the internodal region; (c) photomicrograph of a cross section showing the leaf margin; (d) photomicrograph of a cross section showing the midrib of the leaf; (e) photomicrograph of a cross section showing the vascular system of the petiole. Co – collenchyma, AngCo – angular collenchyma, Cd – crystal (druze), Sc – secretory cavity, Cu – cuticle, AbEp – abaxial epidermis, AdEp – adaxial epidermis, Scl – sclerenchyma, SpP – spongy parenchyma, P – parenchyma, Pp – palisade parenchyma, CrS – crystal series.

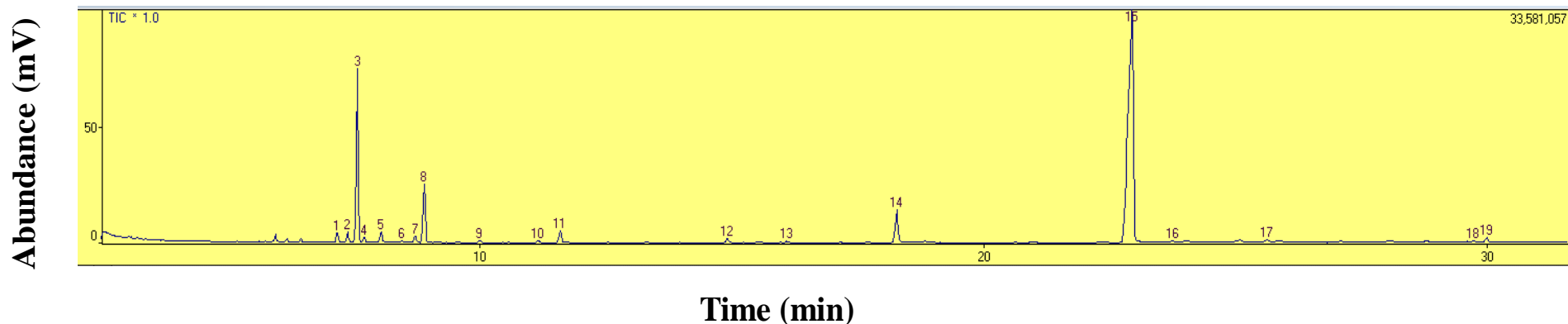


Figure 5. Gas chromatogram of the essential oil of the *Pimenta dioica* leaves from Goiás State, Brazil. For details of the chromatographic conditions, see the Materials and Methods section.

Plant material quality parameters

The samples of *P. dioica* exhibited a moisture content of $7.87 \pm 2.11\%$ (w/w). This value is between the limits that are recommended by the Brazilian Pharmacopoeia V (Brazil, 2010), which established values between 8 and 14% for the moisture content of raw vegetable material. Moisture content tests are important because they reduce errors in estimating the actual weight of the plant material, and low humidity is suggestive of better stability against product degradation. The samples exhibited $8.84 \pm 0.125\%$ (w/w) total ash. The content of HCl-insoluble ash was $1.74 \pm 0.233\%$ (w/w). The ash value is an important parameter for judging the identity and purity of crude drugs (Brazil, 2010).

Phytochemical screening

In addition to the essential oils, the secondary metabolites that were found in the phytochemical

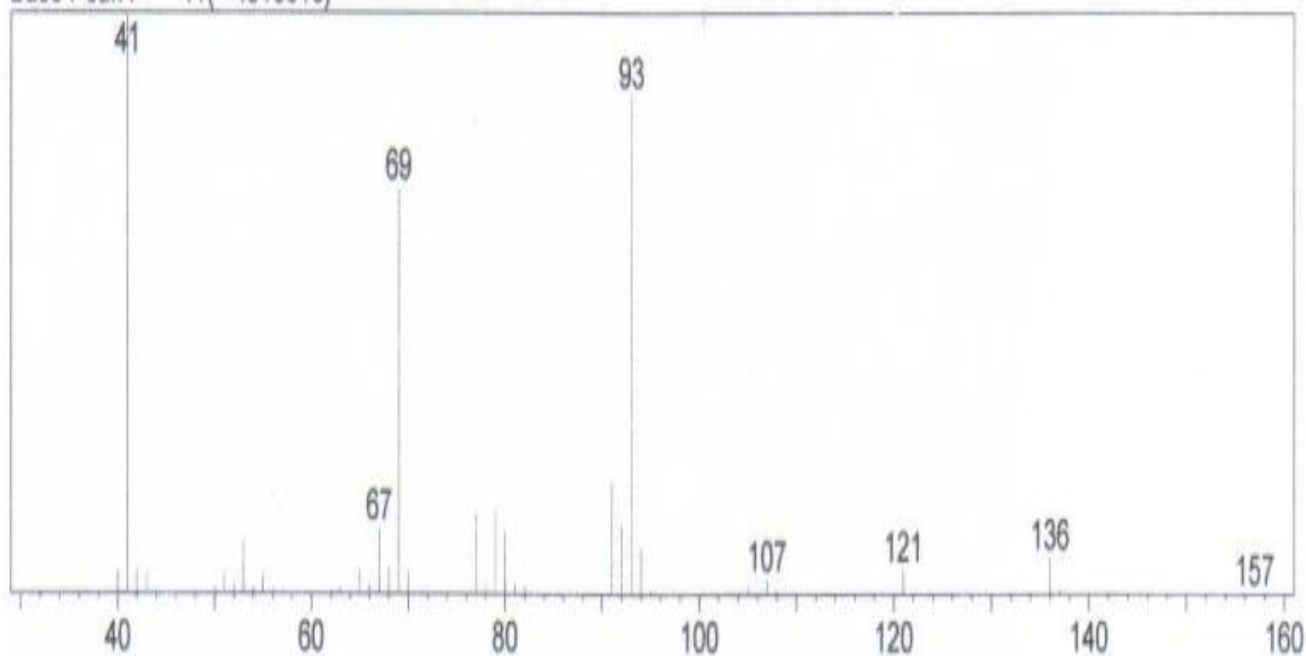
screening of the *P. dioica* leaves included saponins, tannins and flavonoids (Table 1). The total flavonoid found in the samples was $1.88 \pm 0.133\%$ (w/w) as assayed by the method described in the monograph of “Calêndula” (Brazil, 2010). This value is similar to that for *P. pseudocaryophyllus* (Paula et al., 2008). According to Vadlapudi and Kaladhar (2012), *P. dioica* leaves collected from Vijayawada of the Krishna district of India exhibit a flavonoid content of $116.25 \mu\text{g/g}$. These authors attributed the antioxidant and antimicrobial activities that have been observed for *P. dioica* leaves to the high contents of phenolic compounds, including flavonoids, and to the main constituents found in the essential oils. Marzouk et al. (2007) verified the anticancer and antioxidant properties of tannins isolated from *Pimenta dioica* leaves.

Analysis of the essential oils

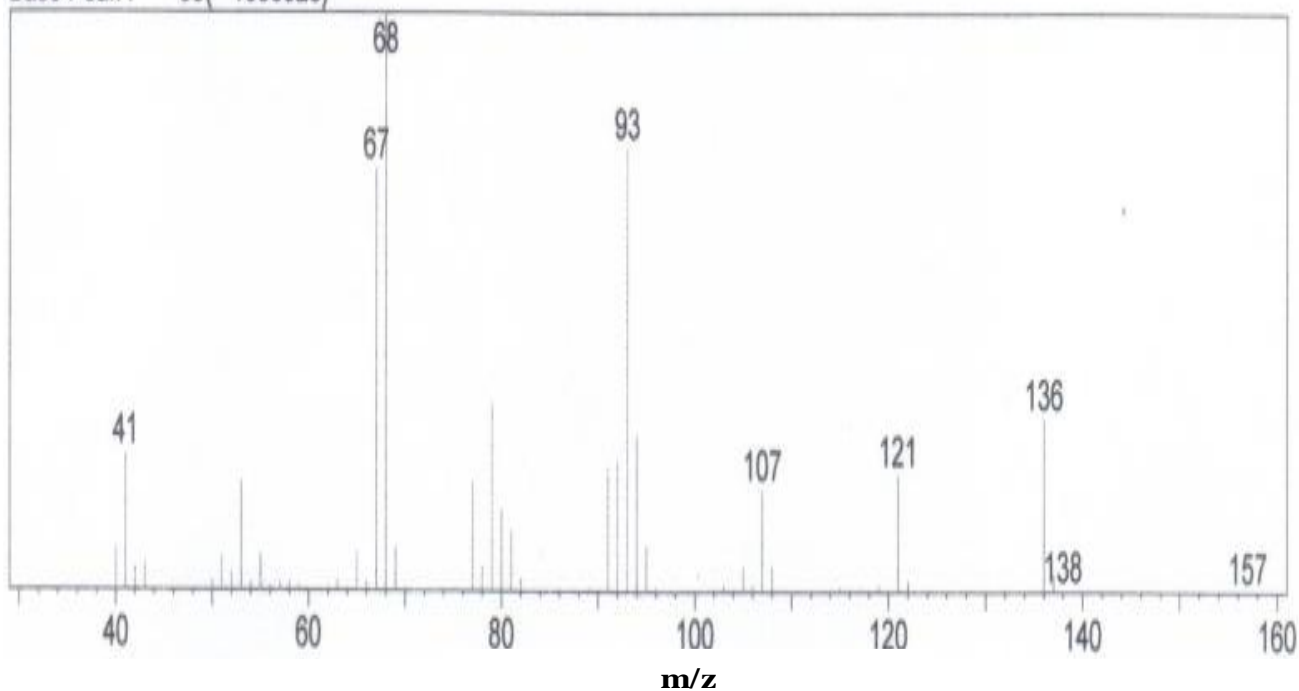
The essential oil yield was 1.3% (v/v), which is

higher than that found in a study of the leaves of sun-dried *P. dioica* specimens that were grown in the southern region of Bahia (Oliveira et al., 2009). The GC/MS analysis of the *P. dioica* leaf-extracted oil identified 19 compounds, and the major components were the phenylpropanoids eugenol (60.8%) and chavicol (4.8%) and the non-oxygenated monoterpenes myrcene (19.3%) and limonene (6.5%; Table 2). The chromatogram is presented in Figure 5, and the mass spectra of the main constituents are represented in Figure 6. These analyses revealed that this botanical material is rich in eugenol and chavicol, which agrees with the report of Mendes-Ferrão (1993). Similar findings have been reported for samples from the southern region of Bahia state in Brazil in which 26 compounds were identified, and the major components were found to be eugenol (78.5%), followed by myrcene (7.3%), β -phellandrene (2.2%) and chavicol (5.5%) (Oliveira et al., 2009). Other authors have identified eugenol as the major component of *P. dioica*

PKNO : 3 Peak #: 86 Ret. Time : 7.583
 Scan # : (305 - 307) B.G. Scan #: (945 - 1201)
 Base Peak : 41(4510016)



PKNO : 8 Peak #: 82 Ret. Time : 8.900
 Scan # : (384 - 386) B.G. Scan #: (945 - 1201)
 Base Peak : 68(1096928)



Relative Abundance

m/z

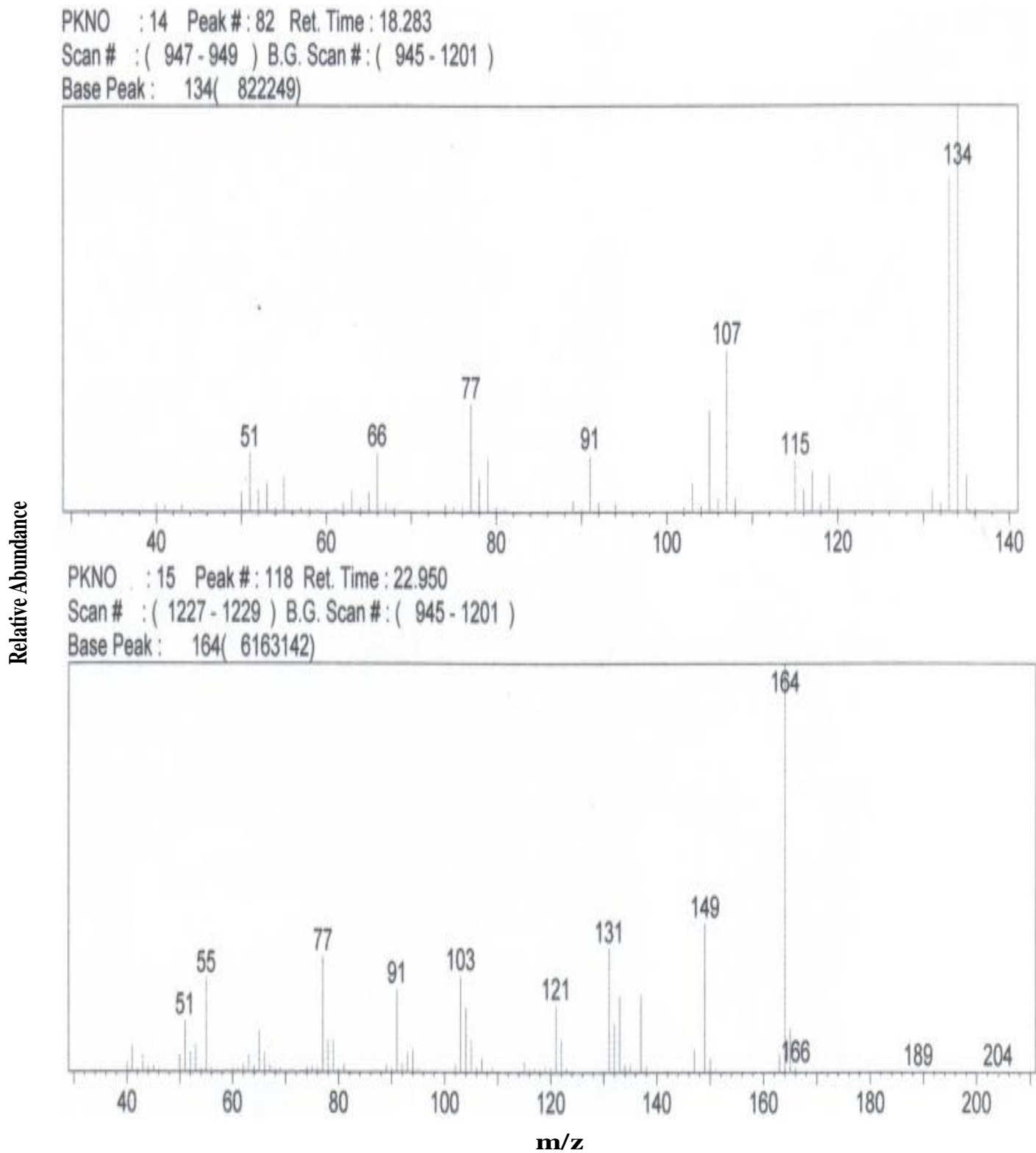


Figure 6. Mass spectra of peaks 3, 8, 14, and 15 of the chromatogram, which indicate the major compounds of the essential oil of the *Pimenta dioica* leaves, which were myrcene, limonene, chavicol, and eugenol, respectively.

Table 1. The phytochemical components of the *P. dioica* leaves based on the preliminary screening.

Phytochemical compound	Occurrence	Total Content (% w/w)
Anthraquinone heterosides	-	-
Digitalis heterosides	-	-
Flavonoids	+	1.88 ± 0.133%
Saponins	+	NP
Tannins	+	NP
Alkaloids	-	-
Coumarins	-	-

+ = presence, - = absence, NP = not performed.

Table 2. Chemical composition of the essential oil from the *Pimenta dioica* (L.) Merrill leaves that were collected in an urban area of Goiás State, Brazil.

Component	KI*	Percentage
1-Octen-3-ol	972	0.97
3-Octanone	979	0.96
Myrcene	986	19.3
3-Octanol	990	0.59
α-Phellandrene	1002	1.19
α-Terpinene	1013	0.17
p-Cymene	1019	0.72
Limonene	1024	6.48
γ-Terpinene	1053	0.20
Terpinolene	1083	0.25
Linalool	1095	1.39
Terpinen-4-ol	1173	0.59
n-Decanal	1200	0.15
Chavicol	1249	4.78
Eugenol	1354	60.8
α-Copaene	1372	0.21
(E)-Caryophyllene	1415	0.35
Myrac aldehyde	1512	0.20
δ-Cadinene	1518	0.68

KI* - Kovats indices.

specimens that were collected in other countries. Marongiu et al. (2005) found that the essential oil of *P. dioica* leaves from Australia is 77.9% eugenol. The leaf oils from fruiting female and non-fruiting male *P. dioica* from Shawbury, St. Ann, Jamaica have been analyzed, and 79.8 to 84.0% eugenol was found (Minott and Brown, 2007). Oussalah et al. (2006, 2007) found that the essential oil of *P. dioica* leaves from Antilles is rich in eugenol (47.78%), myrcene (26.76%) and geraniol (10.40%) and

exhibits antibacterial activity against a *Pseudomonas putida* strain isolated from meat, *E. coli* O157:H7, *Salmonella typhimurium*, *Staphylococcus aureus* and *Listeria monocytogenes*. Vadlapudi and Kaladhar (2012) verified the antioxidant properties of the essential oil of *P. dioica* leaves collected in India and attributed those properties to the high eugenol, methyl eugenol and β-caryophyllene contents (the percentages of each of the constituents was not reported by the authors).

Conclusion

The anatomy of the leaves of the *P. dioica* plants that were grown in Goiás, Brazil exhibited characteristics that were common to other members of the Myrtaceae family and the genus *Pimenta*. The consideration of the anatomical characteristics in conjunction with the morphological characteristics might contribute to the identification of the species and quality control of the medicinal plant. The moisture (7.87%, w/w), total ash (8.84%, w/w), HCl-insoluble ash (1.74%, w/w) and total flavonoids (1.88%, w/w) contents found in the analyzed plant material are important parameters for the quality control of materials which can enable the detection of fraud and tampering. The detection of flavonoids, tannins, eugenol and chavicol in the analyzed botanical materials indicates strong similarities between the chemical compositions of the leaves of *P. dioica* plants that were grown in Goiás, Brazil and those from other parts of Brazil and the world. Additional qualitative and quantitative analyses are necessary to verify the correspondence between the chemical composition of our samples and the biological properties mentioned in the literature.

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Conflict of Interest

Authors have not declared any conflict of interest.

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Review

Taxonomic and phytomedicinal properties of *Oroxylum indicum* (L.) Vent: A wonderful gift of nature

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This paper is a review on pharmacological studies, phytochemical analysis and problems associated with natural seed germination of *Oroxylum indicum* (L.) Vent. The different parts of this plant like leaves, fruits, stem bark, seeds and roots are used in indigenous medicine preparation against various diseases. Chemical investigation of various parts of this plant resulted in characterization of various bioactive principles. It shows antioxidative, antitumour, antiinflammatory, analgesic, antimicrobial and hepatoprotective activity. Due to significant medicinal properties and continuous increasing demand, this plant has been put in endangered category (IUCN).

Key words: *Oroxylum indicum* (L.) Vent, endangered, propagation, medicinal plant, phytomedicinal action.

INTRODUCTION

Over the last thirty years, medicinal plants are 'important' natural resources used by indigenous medicinal system for treatment of diseases. A list of 243 commonly used medicinal plants is drawn having great demand for manufacture of mixtures, compound formation and potent medicine by the Central Council of Research on Ayurveda and Sidha (Thatoi, 2008). The Indian Himalayan Region (IHR) recognized amongst 34 biodiversity hotspots in the world. It contains about 1,748 different species of medicinal plants (Samant et al., 1998).

Among different medicinal plants of IHR, *Oroxylum* (Family: Bignoniaceae) possess high economical, ecological and medicinal importance and has about 112 genera and more than 725 species of trees, shrubs and vines spreading all over the world.

Oroxylum indicum is a small to medium sized deciduous tree widely distributed in tropical and subtropical

regions. This plant is native to the Indian subcontinent, in the Himalayan foothills with a part extending to Bhutan, South China and Malaysia ecozone (Lawania et al., 2010). In India, it is distributed in Eastern and Western Ghats and North East India (Jayaram and Prasad, 2008). North East India comprising the states of Arunachal Pradesh, Assam, Manipur, Meghalaya, Mizoram, Nagaland, Sikkim and Tripura lying between 21°34'N to 29°50'N latitude and 87°32'E to 97°52'E longitude (Mao et al., 2009).

The English name of this plant is "Indian trumpet flower" and commonly known as "Shyonaka". It grows upto 10 to 12 m with light grayish brown, soft and spongy bark, in its natural habitat. Leaves are compound, 60 to 120 cm in length, pinnate and stipules are absent. The flowers are bisexual and zygomorphic. Androecium consists most commonly of didynamous stamens that are attached to the corolla tube. The gynoecium consists of a

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single compound pistil of two carpels, a single style and a superior ovary with typically two locules, each bearing numerous axile ovules. An annular or cupular nectary disk is usually found around the base of ovary. The fruit is capsular with winged seeds or sometimes indehiscent with wingless seeds (Angiosperm phylogeny website, Wikipedia). *O. indicum* is chiropterophilous in nature, and while studying its pollination ecology it has been reported that dawn bat *Eonycteris spelaea* is the pollinator of *O. indicum* in Western Malaysia and *Leschenault's Roussette* (*Rousettus leschenaultii*) as a pollinator in India (Fujita, 1991).

O. indicum lives in relationship with an actinomycete *Pseudonocardia oroxyli*, a high-G+C-content, Gram-positive bacterium, strain D10T present in the soil surrounding the roots. Based on 16S rRNA gene sequence analysis, strain D10T was a member of the genus *Pseudonocardia* and was most closely related, albeit loosely, to *Pseudonocardia halophobica*. (Gu et al., 2006). Numerous studies have indicated that these prolific actinobacteria appear to have a capacity to produce an impressive array of secondary metabolites exhibiting a wide variety of biological activity, such as antibiotics, antitumor and anti-infection agents, plant growth promoters and enzymes, and may contribute to their host plants by promoting growth and enhancing their ability of withstanding the environmental stresses (Qin et al., 2011). In nature, different developmental stages of *O. indicum* are shown in Figure 1.

Therefore, this study was done to evaluate the different medicinal properties of *O. indicum* and to explore the need of conserving this plant.

CHEMICAL CONSTITUENTS OF *O. INDICUM*

The plant *O. indicum* contains flavonoids like chrysin, oroxyline, and baicalein as active principal component (Choudhury et al., 2011). From the seeds of *O. indicum*, amounts of bioactive flavonoids such as baicalein-7-o-diglucoside, baicalein-7-o-glucoside, baicalein, chrysin, and apiagenin have been identified (Chen et al., 2003). Flavonoids such as chrysin, oroxylin-a, scutellarin, baicalein, biochanin-a and ellagic acid shows anti-arthritis, diuretic, antifungal, anti-inflammatory and anti-bacterial activities (Zaveri et al., 2008). Chemical investigations of acetone and hexane extracts of stem bark resulted in isolation and characterization of two new flavonoids which are found to possess gastro-protective activity (Babu et al., 2010). The root extracts of *O. indicum* inhibited the activity of both enzymes-5-lipoxygenase and cyclooxygenase (Ali et al., 1998). Seeds of this plant are also reported to contain ellagic acid, which is an important polyphenolic compound (Sastry et al., 2011). Flavones, sterols and prunetin have been reported in different parts of *O. indicum* (Chen et al., 2003). Different phytochemical and their quantitative

values are as shown in Table 1.

TRADITIONAL/FOLK USES

“Shyonaka” is a wonderful plant because almost every part such as leaves, stem bark, root bark, and fruits possess medicinal properties. It has been used in several traditional Ayurvedic and Folk medicines. The root bark of this plant is reported to be administered as astringent, bitter tonic, stomachic and anodyne. The root bark is an important ingredient in famous tonic formulations such as Chyawanprash, Dashmularisht, Narayan taila, Bhrama Rasayana, Dhanwatarra, etc., (Sastry et al., 2011).

The decoction of the bark is taken for curing gastric ulcer and a paste made of the bark powder is applied for mouth cancer, scabies and other skin diseases. The seed is ground with fire-soot and the paste applied to the neck for quick relief of tonsil pain. Also, a paste made of the bark is applied to the wounds of animals to kill maggots. Decoction of the bark is given to animals for de-worming. The sword-like fruit or a branch of the plant is used by the farmers to kill crabs in wet paddy fields (National Innovation foundation-India). Mature fruits are acrid, sweet, anthelmintic, and stomachic. They are useful in pharyngodynia, cardiac disorders, gastropathy, bronchitis, haemorrhoids, cough, piles, jaundice, dyspepsia, smallpox, leucoderma and cholera (Warrier et al., 1995).

Seeds are used as purgative. Dried seed powder is used by women to induce conception. Seeds yield non-drying oil used in perfume industry. The seeds are ground with fire soot and the paste is applied to the neck for quick relief of tonsil pain. The medicated oil of *O. indicum* in sesame oil base instilled into ears mitigates the pain in otitis (Chauhan, 1999). Roots are sweet, astringent, acrid, refrigerant (Yoganarasimhan, 1996), aphrodisiac, expectorant, carminative, digestive, anthelmintic, constipating, diaphoretic, diuretic, antiarthritic, anti-diabetic and febrifuges. Tonic is useful in dropsy, cough, sprains neuralgia, hiccough, asthma, bronchitis, anorexia, dyspepsia, flatulence, colic, diarrhea, strangury, gout, vomiting, leucoderma, wounds, rheumatoid arthritis and fever. Root bark is used in stomatitis, nasopharyngeal cancer and tuberculosis (Khare, 2004; Bhattacharje, 2005).

It is believed to have been prescribed by Malayasians to treat toothache, wound, splenomegaly, gastralia, dysentery, cholera, loss of appetite and fever. Any part of the plant may be used for making a decoction for external uses in childbirth. In Philippine Islands, the bark of the root is claimed as a diaphoretic. An alcoholic maceration of fresh bark is applied externally as a lacquer to relieve allergic dermatitis (Herbal Medicine Research Institute, 2002). In Vietnamese folk medicine, a decoction of the seeds is used for cough, bronchitis and gastritis.

In China, the seeds of *O. indicum* are used to cure liver and stomach problems and to heal ulcers and boils. In

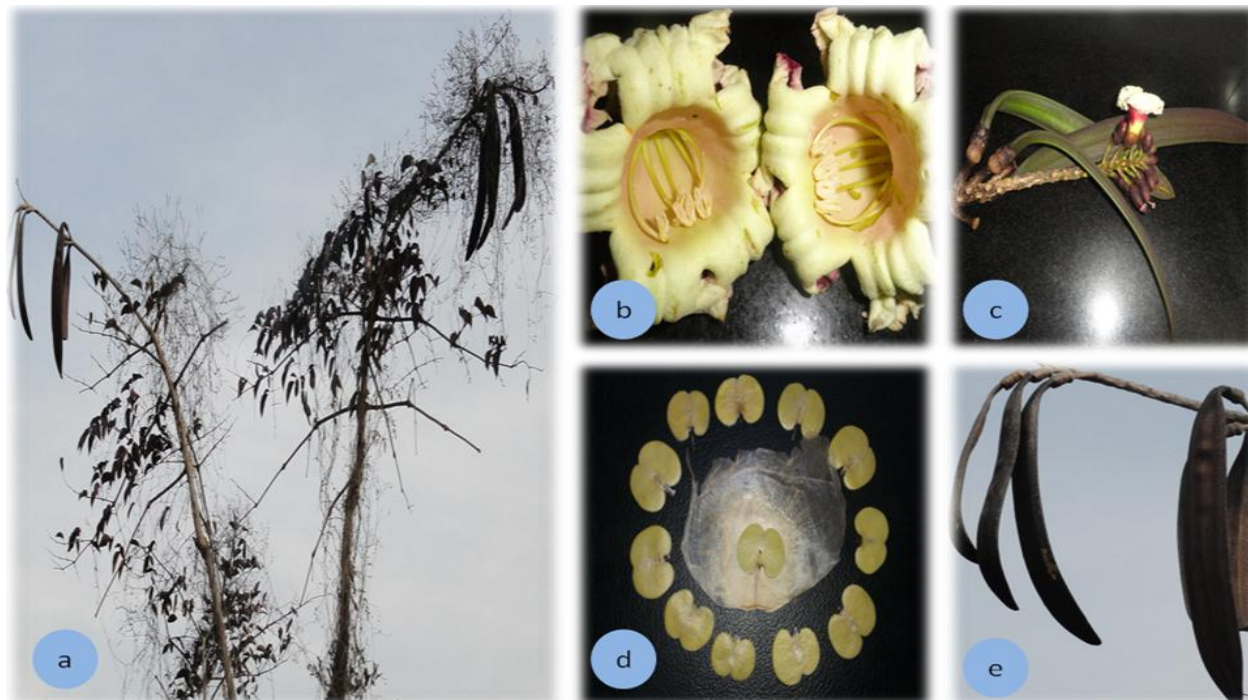


Figure 1. Different developmental stages of *O. indicum*: (a) Tree growing in its natural habitat (photograph taken from village Fatehpur, Haldwani (Uttarakhand) in the month of March, (b) Flowering during the month of August, (c) Flower and fruits, photograph taken in month of August, (d) Winged and naked seeds, and (e) Capsular fruits.

Table 1. Quantitative phytochemical values (D'Mello et al., 2012).

Total tannins	Total glycosides	Total phenolics dry weight	Total phenolic acids mg/g dry weight
10.63% w/v	12.95% w/v	1.436	1.275 ± 0.062

Burma, Vietnam and Philippines, the bark is used to treat dysentery and rheumatism (Choudhury et al., 2011).

PHARMACOLOGICAL USES

O. indicum seeds, leaves, stem bark and root bark are used in treatment of remittent fever, otorrhoea, bronchitis, leucoderma, diarrhea, inflammation and acute rheumatism (Bisht et al., 2011; Sastry et al., 2011). Various medicinal properties are shown Table 2. The different chemical extracts from stem bark, fruits and roots have different pharmacological activities.

Antitumor activity

Root bark of *O. indicum* shows antiproliferative activity on HL-60 cell line and anticancerous on CEM, B-16 and HCT-8 cell lines (Thatoi et al., 2008). Baicalein, the most abundant flavonoid present in the leaves of *O. indicum* has been isolated and tested on the viability and induction of apoptosis in the HL-60 cell line. Pretreatment

with baicalein for 24 h caused a 50% inhibition of HL-60 cells at concentrations of 25 to 30 μM exposure of HL-60 cells to 10 to 20 μM baicalein for 36 to 48 h caused the cells to accumulate at S or G2M phases. The result of the study indicated that the baicalein has anti-tumor effect on human cancer cells (Roy et al., 2007). Non-polar extracts of *O. indicum* (especially PHO, petroleum ether hot extract) can effectively target Estrogen Receptor (ER)-negative breast cancer cells to induce apoptosis, without harming normal cells by cancer-specific cytotoxicity. Hence, it could be considered as an extract with candidate precursors to possibly harness or alleviate ER-negative breast cancer progression even in advanced stages of malignancy (Kumar et al., 2012).

The antitumor property of *O. indicum* has been evaluated in the experimental animals induced by different types of carcinogens, and in human cell lines by a number of experiments. Ethanolic extract of *O. indicum* was found to have antiproliferative effect on Hep 2 cell lines. Ethanolic extract exhibited cytotoxic activity against the Hep 2 cell lines at a concentration of 0.05% (Narisa et al., 2006).

Table 2. Major constituents of *O. indicum* and their activity (Zaveri et al., 2008; Choudhry et al., 2011).

Flavonoid	Concentration (µg/ml)	Activity
Baicalein	8.0	Antioxidant, antimutagenic, anticancerous, and antiinflammatory
Chrysin	8.0	Antioxidant, nephroprotective, and immunomodulatory
Biochanin A	26.0	Antidiuretic, antimicrobial, antiarthritic, and antioxidant
Ellagic acid	8.0	Antioxidant, hepatoprotective, and antifungal

Antioxidant activity

The production of different oxidative species and free radicals due to stress leads to adverse effects on various vital organs and tissues of body. Antioxidants are now standing on the mainstay of the treatment and prevention of several diseases (Uttara et al., 2009). Current research is directed towards finding naturally occurring antioxidants particularly of plant origin. *In vitro* antioxidant activity of n-butanol extract of stem and root bark of *O. indicum* was determined by 2, 2'-Azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS) radical cation decolorization assay and reveals the presence of very high significant antioxidant activity (Zaveri and Dhru, 2011). *In vitro* antioxidant activity (IC₅₀ 22.7 µg/ml) was the highest in methanolic extract of stem bark of *O. indicum* (Moirangthem et al., 2013). The ethanol extract of stem bark exhibited maximum antioxidant potential due to its free radical scavenging activities in b-carotene bleaching assays, whereas chloroform extract showed maximum reducing power in total antioxidant activity may be due to its reducing potential (Kalaivani and Mathew, 2009). The ethyl acetate extract (EAE) of stem bark showed the lowest IC₅₀ value (0.76 mg/ml) with the highest inhibition of 2, 2-diphenyl-1-picrylhydrazyl (DPPH). In the ferric reducing antioxidant power (FRAP) assay, the hexane extract (HE) showed the highest ferric reducing ability whereas the EAE showed better antioxidant ability with the lowest IC₅₀ value (0.80 mg/ml) (Kumar et al., 2011). Methanolic and aqueous extracts of stem bark of *O. indicum* have also been found to have diverse therapeutic potentials. Various properties including antioxidant property, cytotoxicity, and protection against oxidative DNA damage, FRAP, free radical (DPPH and OH⁻) scavenging activities as well as inhibitory effect on lipid peroxidation have also been confirmed. In the cytotoxicity test, cytotoxicity of the extracts has been characterized by XTT assay in MDA-MB-435 S and Hep 3D cell lines. Protection of DNA by the extracts against oxidative damage by UV-photolysis of H₂O₂ was studied. Both extracts inhibited lipid peroxidation in a dosage dependent manner. Both extracts exhibited considerable free radical scavenging and ferric reducing abilities. The extracts demonstrated extensive cytotoxicity in both tested cell lines. Both extracts exhibited moderate levels of DNA protection against oxidative stress (Ahad et al., 2012).

Antiinflammatory activity

O. indicum is found to possess an efficient anti-inflammatory activity. Dichloromethane extract of the stem bark and root of *O. indicum* were found to have anti-inflammatory properties (Ali et al., 1998). Different extracts of *O. indicum* against experimental acute and chronic inflammatory model has proven the occurrence of anti-inflammatory activity in the plant extracts. *O. indicum* showed most promising NF-kappa B inhibitory effect with the lowest IC (50) value and is identified as Thai anti-inflammatory remedy (Siriwatanametanon et al., 2010). Arya and Arya (2011) reported that *O. indicum* have flavones and glycosides such as baicalein and scutellarin which imparts anti-inflammatory effect to its bark.

The antiinflammatory activity was evaluated by carageenan induced rat paw edema model in rats using diclofenac sodium as standard drug. Two doses 150 and 300 mg/kg of aqueous extract of *O. indicum* were used. Result showed that paw volume was significantly reduced in dose dependent manner as compared to control. Extract at a dose of 300 mg/kg showed maximum antiinflammatory activity. However, the activity produced by both the doses was less than the reference standard. Extract at both doses showed significant (P<0.05) anti-inflammatory activity at 5 h suggesting that the extract predominantly inhibit the release of prostaglandin like substances (Upaganlawar et al., 2009).

Antimicrobial activity

The anti-microbial activity of various extracts of *O. indicum* has been screened against various pathogens and reveals the use of plant extract and phytochemicals for therapeutic treatment (Das and Chaudhry, 2010). The antimicrobial activity of crude extract of *O. indicum* (petroleum ether, ethyl acetate and methanol), compound 1 (2,5-dihydroxy-6,7-dimethoxy flavone) and compound 2 (3, 7, 3', 5'-tetramethoxy-2-hydroxy flavone) was tested against fourteen pathogenic bacteria (5 Gram-positive and 9 Gram-negative) and seven pathogenic fungi. Nutrient agar and nutrient broth were used as bacteriological media and potato dextrose agar (PDA) was used for fungal growth. In antibacterial screening, each sample was dissolved in methanol at a concentration of 200 µg/10 µl. The activity of these

samples was compared with standard kanamycin disc (K-30 µg/disc) using the standard disc diffusion method. Similarly, antifungal screening was done at a concentration of 300 µg/disc for each sample and the activity was compared with the standard clotrimazole disc (K-30 µg/disc). From the antibacterial and antifungal experimental results, it was evident that the crude extracts (petroleum ether, ethyl acetate) and compounds 1 and 2 showed significant antibacterial and antifungal activity, but were less potent than that of standard kanamycin and clotrimazole, whereas methanol extract showed little activity. The findings support the use of *O. indicum* in traditional medicine for the treatment of bacterial and fungal infection (Chopade et al., 2008). The antimicrobial activity of 40 medicinal plants has been examined from Similipal Biosphere Reserve, Orissa, India. *O. indicum* was one of those plants with prominent antimicrobial activity in root bark against various pathogenic bacteria e.g., *Staphylococcus aureus*, *Bacillus licheniformis*, *Bacillus subtilis*, *Pseudomonas aeruginosa*, *Vibrio cholerae*, etc., (Thatoi et al., 2008). Dichloromethane extracts of stem bark and root of *O. indicum* were found to have antimicrobial activities against Gram-positive (*B. subtilis* and *S. aureus*), Gram-negative (*Escherichia coli* and *P. aeruginosa*) and a Yeast (*Candida albicans*) (Ali et al., 1998). Uddin et al. (2003) isolated two flavonoid from *O. indicum*- 2,5-dihydroxy-6,7-dimethoxy flavones (1) and 3,7,3',5'-tetramethoxy-4'-hydroxy flavones (2). The MIC of compounds 1 and 2 were measured against *B. subtilis*, *S. aureus*, *E. coli* and *Shigella dysenteriae* and values were found between 64 and 128 µg/ml.

Immunomodulatory activity

The root bark of *O. indicum* was found to have immunostimulant/immunomodulatory activity (Zaveri et al., 2006). Research showed that treatment with n-butanol fraction of the root bark of *O. indicum* resulted in significant rise in circulating hemagglutinating antibody titers during secondary antibody responses, represents the potentiation of certain aspects of the humoral response. The treatment also resulted in a significant rise in paw edema formation, indicating increased host delayed type hypersensitivity (DTH) response. Additionally, the antioxidant potential of the drug was exhibited by significant reductions in whole blood malondialdehyde (MDA) content along with a rise in the activities/levels of superoxide dismutase (SOD), catalase (CAT) and reduced glutathione (GSH). In addition, histopathologic analysis of lymphoid tissues showed an increase in cellularity, e.g., T-lymphocytes and sinusoids, in the treatment group. The reported immunomodulatory activity of an active fraction of *O. indicum* might be attributed to its ability to enhance specific immune responses (both humoral and cell-mediated) as well as

antioxidant potential.

The immunomodulatory properties of *O. indicum* have also been evaluated through estimation of humoral and cell mediated immune response in broiler chicks. Day old broiler chicks were grown up to 7 days and divided into different groups. All the groups were vaccinated against Ranikhet disease (RD) virus on days 7 and 28 except one group which served as unvaccinated control. All the groups of chicks were vaccinated against infectious bursal disease (IBD) on day 14. Two groups of chicks were fed *O. indicum* stem bark and root bark powder, respectively each at 250 mg/kg body weight. One group was fed levamisole at 10 mg/kg body weight, which acts as standard drug. Humoral immune response was measured by hemagglutination (HA) test and hemagglutination inhibition (HI) test against ND virus. Cell mediated immune response was studied on the basis of delayed hypersensitivity reaction or measured by contact sensitivity test with 2, 4-dinitrofluorobenzene (DNFB). The highest MHI antibody titer was found in levamisole treated group, followed by groups treated with root powder and stem powder of *O. indicum*, respectively. Similarly, six hours post DNFB challenge, a significant rise in mean skin thickness (MST) was observed in all the treated groups. The highest was observed in levamisole treated group, followed by groups treated with root powder and stem powder of *O. indicum*, respectively. These findings suggested that the root bark of *O. indicum* possessed the significant immunomodulatory activity, that is stem bark counterpart. Thus, *O. indicum* root bark powder may be recommended as safe and commercially beneficial immunomodulator (Kumari et al., 2011).

Hepatoprotective activity

Hepatoprotective activities of petroleum ether, chloroform, methanolic and aqueous extracts of *O. indicum* were examined against carbon tetrachloride induced liver damage in mice using silymarin as control. These studies indicate that alcoholic stem bark extracts of *O. indicum* had activity over carbon tetrachloride treatment as compared to control. Results of the present investigation confirmed the traditional use of this plant as a potential hepatoprotective agent (Tripathy et al., 2011). *O. indicum* has been found to offer liver protection against various experimentally induced damages. Different extracts of leaves of *O. indicum* showed significant hepatoprotective activity against CCl₄ induced hepatotoxicity in Wistar albino rats. Carbon tetrachloride injection leads to the significant increase in the level of serum enzymes. Ethanolic extract was found to be more effective than all other extracts (Siddiqui et al., 2012). The aqueous root extract of *O. indicum* has potent hepatoprotective activity against paracetamol induced liver damage in rats, it also showed that, it has great influence on liver blood parameters. Thus, it is proved

that *O. indicum* aqueous extract has potent hepatoprotective activity and was found to be dose dependant and this could be attributed to the new flavonoid Oroxylin-B (Sastry et al., 2011). The hepatoprotective activity of *O. indicum* was studied against carbon tetrachloride (CCl₄)-induced hepatotoxicity in mice and rats. Biochemical study indicated that alcoholic (300 mg/kg), petroleum ether (300 mg/kg) and *n*-butanol (100 and 300 mg/kg) extracts significantly ($P < 0.05$) lowered the elevated serum glutamic oxaloacetic transaminase (SGOT), serum glutamic pyruvate transaminase (SGPT), alkaline phosphatase (ALP) and total bilirubin (TB) levels as compared to the control group. The increased lipid peroxide (LPO) formation, reduced glutathione (GSH) and decreased antioxidant enzyme activities of superoxide dismutase (SOD), catalase (CAT) in the tissues of CCl₄-treated animals were significantly normalized by *O. indicum* treatment. Histopathological study also revealed that pretreatment with *O. indicum* restored CCl₄-induced alteration in antioxidant status of the tissues. It is suggested that root bark showed significant antioxidant activity, which might be in turn responsible for its hepatoprotective activity (Zaveri and Jain, 2009). The hepatoprotective activity of stem bark of *O. indicum* against CCl₄ induced liver damage in mice has also been confirmed. Pet ether, chloroform, methanolic and aqueous extracts of stem bark of *O. indicum* were examined against carbon tetrachloride induced liver damage in mice using silymarin as control. Enzyme activities of SGPT, ALP and SGOT were analyzed. All the extracts were shown to have significant hepatoprotective activity, with the methanolic extract being more efficient (Bichitra et al., 2011).

Antimutagenic activity

Methanolic extract of *O. indicum* strongly inhibited the mutagenicity of Trp-P-1 in Ames test. The major antimutagenic constituent was identified as baicalein with an IC₅₀ value of 2.78±0.15 µM. The potent antimutagenicity of the extract was correlated with the high content (3.95±0.43%, dry weight) of baicalein. Baicalein acted as a desmutagen since it inhibited the N-hydroxylation of Trp-P-2 (Nakahara et al., 2001). Methanolic extract of the fruits of *O. indicum*, inhibited *in vitro* proliferation of HL-60 cells and flavonoid baicalein was found as an active component in the extract (Roy et al., 2007). *In vitro* anti-mutagenic activity of selected plants including *O. indicum*, was done by Ames *Salmonella* mutagenicity test using histidine mutants of *Salmonella typhimurium* tester strains, MTCC 98, MTCC 1251 and MTCC 1252. The hydroalcoholic extract of *O. indicum* significantly inhibited ($P < 0.001$) the *in vitro* by direct mutagens-sodium azide (NaN₃), 4-nitro-o-phenylenediamine (NPD), and indirect mutagens benzo[a]pyrene (B[a]P) 2-aminoflourene(2-AF) induced

his revertants in a dose dependent manner (Zaveri et al., 2011). Ethanol soluble fraction prepared from twigs and leaves of this plant have reported for their antimutagenic activity (Wall et al., 1988). Methanolic extracts have antimutagenic activity against heterocyclic amines (ayurvedainstitute.org/shop/MNOMedicines.htm).

Gastroprotective activity

The *n*-butanol fraction of the root bark of *O. indicum* showed significant gastroprotective activity against both ethanol and Water Immersion Plus Restraint Stress (WIRS)-induced gastric ulcers in rats, that could be attributed to its antioxidant activity, vasodilatation, and gastric cytoprotection (Zaveri and Jain, 2007). Chemical investigation of the stem bark of *O. indicum* resulted in the isolation and characterization of two new flavonoid glycosides. Their structures were established on the basis of extensive spectroscopic (IR, MS, 2D NMR) data analysis, and all the compounds were tested for their ulcer protective effects against various gastric ulceritis inducing models in rats (Babu et al., 2010). Various polyphenolic compounds have been reported for their anti-ulcerogenic activity with a good level of gastric protection. Besides their action as gastroprotective, these phenolic compounds can be an alternative for the treatment of gastric ulcers. Therefore, considering the important role of polyphenolic compounds in the prevention or reduction of gastric lesions induced by different ulcerogenic agents, in this review, we have summarized the literature on some potent antiulcer plants, such as, *O. indicum*, *Zingiber officinale*, *Olea europaea* L., *Foeniculum vulgare*, *Alchornea glandulosa*, *Tephrosia purpurea*, etc., containing phenolic compounds, namely, baicalein, cinnamic acid, oleuropein, rutin, quercetin, and tephrosin, respectively, as active constituents (Sumbul et al., 2011).

The 50% alcohol extract of the root bark of *O. indicum* and its petroleum ether, chloroform, ethyl acetate and *n*-butanol fractions were studied against ethanol-induced gastric mucosal damage. The alcohol extract (300 mg/kg, p.o.) and its different fractions (100 and 300 mg/kg, p.o.) showed reduction in gastric ulceration. The petroleum ether and *n*-butanol fractions showed maximum inhibition of gastric lesions against ethanol-induced gastric mucosal damage. The results were comparable with omeprazole (reference standard). In the ethanol-induced gastric ulcer model, treatment with both the active fractions and omeprazole showed significant antioxidant activity as evident from the reduction in the extent of lipid peroxidation. The effect of active fraction of root bark on the ulcer index, total acidity, total acid output, pepsin activity, pepsin output and total carbohydrate to protein ratio in pyloric-ligated rat was studied. The active fraction of root bark at a dose level of 100 mg/kg p.o. showed significant reduction ($P < 0.05$) in

the ulcer index, total acidity, total acid output, pepsin activity and pepsin output along with a significant rise in total carbohydrate to protein ratio. The mechanism of antiulcer activity could be attributed to a decrease in gastric acid secretory and antioxidant activities leading to gastric cytoprotection. This activity could be linked to the presence of baicalein in the root bark of the plant (Khandhar et al., 2006).

Commercial value

O. indicum has a number of medicinal properties and various parts of this plant have been used in Ayurvedic preparations. The large pods of this plant sold at a market downtown, Bangkok, Thailand. The tree is often grown as an ornamental for its strange appearance. Materials used include the wood, tannins and dyestuffs (Wikipedia, 2013). Extracts of this plant are also used in herbal tea formulations like vata and kapha (BF1.biz, 2009). *O. indicum* is also used in mentat (mindcare) and mentat syrup preparation (La Medica, 2007).

PROBLEMS ASSOCIATED WITH THE PLANT

According to the Red list of threatened species, 44 plant species are critically endangered, 113 are endangered, and 87 vulnerable (IUCN, 2000). Many medicinal plants are also in trouble due to over harvesting and destruction of habitat. *O. indicum* has become 'vulnerable' in some states of India due to reduction of its population (Sharma et al., 2010). It has also been listed to 'endangered' category in some other states (Gokhale and Bansal, 2005; Tewari et al., 2007). This plant naturally germinate by seeds, in the beginning of rainy season; however, the seed set is poor and seed viability is low, are the problems for its natural propagation along with its indiscriminate exploitation for medicinal purpose have pushed it to this state.

CONCLUSION

Enormous uses of *O. indicum* in medicine and Ayurvedic preparations revealed its properties as antimicrobial, antimutagenic, antiinflammatory, gastroprotective, etc. Therefore, the plant has effective pharmacological action and has proven its potential for future researchers but still the antitumor property of this plant needed to be investigated and further studies may be carried out to prove its promising uses. Due to high medicinal value of this plant, there has been increasing pressure on the collection of this species. So, altitudinal characterization of genetic diversity for this plant shall be helpful for conservation strategies. *In vitro* propagation method offers highly efficient tool for mass multiplication of many threatened plants. Hence, there is an urgent need to

develop efficient and rapid conservation strategy for this plant.

Conflict of Interest

Authors have not declared any conflict of interest.

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Full Length Research Paper

An investigation of the hepatoprotective potential of *Garcinia kola* seed extract in an anti-tubercular treatment model

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Hepatoprotective effect of *Garcinia kola* seed extract on oxidative stress and hepatotoxicity biomarkers of rats exposed to antitubercular drugs was investigated to evaluate its potential to ameliorate drug-induced hepatotoxicity. Six groups of five animals each were used. Combination of Isoniazid (7.5 mg/kg bodyweight) and Rifampicin (10 mg/kg bodyweight) was administered intraperitoneally to a group. Drug combination was co-administered with *G. kola* seed extracts (40, 60 and 80 mg/kg bodyweight) to three other groups for 35 days. Control group received saline and the last group received *G. kola* alone (60 mg/kg bodyweight). Plasma oxidative stress biomarkers (superoxide dismutase, glutathione, glutathione peroxidase, catalase and malondialdehyde) and hepatotoxicity biomarkers (alanine aminotransferase, aspartate aminotransferase and alkaline phosphatase) were assayed after treatment. The antitubercular drugs significantly increased levels of hepatotoxicity biomarkers. The increase was mitigated by treatment with *G. kola*, with reduction in levels of the hepatotoxicity biomarkers upon *G. kola* co-administration. Similarly, the antitubercular drugs reduced the activities of oxidative stress biomarkers. Histomorphological analysis of the liver showed that the *G. kola* extract administered at 60 mg/kg offered significant protection from exposure to the hepatotoxic drugs. The study concluded that *G. kola* extract at 60 mg/kg significantly protected the animals from the damages occasioned by exposure to hepatotoxic antitubercular drugs.

Key words: Isoniazid, rifampicin, tuberculosis, hepatotoxicity, hepatoprotection, histomorphology.

INTRODUCTION

Tuberculosis (TB) remains a major health concern worldwide. Nearly a third of the world's population is

infected with TB, and in 2012, there were around 1.3 million TB-related deaths worldwide (Center for Disease

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Control (CDC), 2014). The causative organism *Mycobacterium tuberculosis* is an ancient pathogen in humans, having been associated with humans since antiquity (Leung et al., 2013). *M. tuberculosis* attacks the lungs mainly, but can also affect other parts of the body when the microbes gain entry into the bloodstream from an area of damaged tissue. TB – a contagious and air borne disease affecting young adults mostly in their productive years, ranks the second leading cause of death from an infectious disease worldwide (World Health Organization (WHO), 2012). The bacteria spread through the air when people who have an active TB infection cough, sneeze or otherwise transmit their saliva through the air (Konstantinos, 2010).

Social factors that are known to be driving the global increase in TB cases include: overcrowding (especially in urban centers), late reporting and diagnosis, non-compliance to treatment schedule, lack of commitment on the part of National control programmes in developing countries, lack of education, health care infrastructure and poverty (Collins and Ahorlu, 2013). The bacteria multiply in organs that have oxygen pressure such as the upper lobe of the lungs, the kidneys, the central nervous system (in tuberculous meningitis) etc. (Kabra et al., 2006).

TB treatment is difficult and requires administration of multiple antibiotics over a long period of time. Recommended standard treatment for adult respiratory TB is a regimen of Isoniazid (INH), Rifampicin (RIF), Pyrazinamide and Ethambutol for 2 months followed by 4 months Isoniazid and Rifampicin alone (WHO, 2003). The most frequent adverse effects of antituberculosis treatment are hepatotoxicity, skin reactions, gastrointestinal and neurological disorders.

A previous study in our laboratories showed that combined INH/RIF administration for 15 days in adult Wistar rats produced astrocytic aggregation and hyperactivity in the cerebral cortex (Fakoya et al., 2013). Hepatotoxicity, however, is the most serious adverse effect (Frieden et al., 2003). INH hepatotoxicity is a common complication of antituberculosis therapy that ranges in severity, from asymptomatic elevation of serum transaminases to hepatic failure requiring liver transplantation. INH is metabolized to monoacetyl hydrazine, which is further metabolized to a toxic product by cytochrome P450 leading to hepatotoxicity.

The combined use of RIF and INH has been associated with an increased risk of hepatotoxicity (Tostmann et al., 2008). Rifampicin induces isoniazid hydrolase, increasing hydrazine production when rifampicin is combined with isoniazid (especially in slow acetylators), which may explain the higher toxicity of the combination. Rifampicin also interacts with anti-retroviral drugs and affects the plasma levels of these drugs as well as risk of hepatotoxicity (Tostmann et al., 2008). Antituberculosis drug-induced hepatotoxicity (ATDH) causes substantial morbidity and mortality and diminishes treatment effectiveness. Peroxidation of in the cytotoxic action of

INH and RIF (Lian et al., 2013). The mechanism is generally attributed to the formation of the highly reactive oxygen species (ROS) which act as stimulators of lipid peroxidation and the source for destruction and damage to the cell membrane (Lian et al., 2013). Asymptomatic transaminase elevations are common during antituberculosis treatment but hepatotoxicity can be fatal when not recognized early and when therapy is not interrupted in time (Kaona et al., 2004). Adverse effects diminish treatment effectiveness because they significantly contribute to non-adherence, eventually contributing to treatment failure, relapse or the emergence of drug-resistance (Kaona et al., 2004). Adherence to the prescribed treatment is crucial for curing patients with active TB (Wares et al., 2003).

Garcinia kola (Heckel), commonly called bitter kola, is an angiosperm, belonging to the family Guttiferae. Bitter kola is a highly valued ingredient in African ethnomedicine because of its varied and numerous uses which include the social and medicinal; thus making the plant an essential ingredient in folk medicine. Medicinal plants such as *G. kola* are believed to be an important source of new chemical substances with potential therapeutic benefits (Adesuyi et al., 2012). The seed is commonly used as a masticatory and is a major kola substitute offered to guests at home and shared at social ceremonies. The seeds are used in folk medicine and in many herbal preparations for the treatment of ailments such as laryngitis, liver disorders and bronchitis (Farombi, 2003). Some of the phytochemicals compounds that have been isolated from *G. kola* include: oleoresin, tannin, saponins, alkaloids, cardiac glycoside, biflavonoids such as kolaflavone and 2-hydroxyflavonoids, and kolavirons (Adesuyi et al., 2012).

Tuberculosis is a health risk for a large number of people. The current treatment regimens are long and pose adverse effects, among which is hepatotoxicity. This has increased the level of non-compliance to treatment in patients. *G. kola* has been shown to possess hepatoprotective and antioxidative properties. This study was designed to evaluate the possibility of utilizing the extracts of this popularly eaten plant to alleviate the hepatotoxicity associated with these antitubercular drugs, thus encouraging compliance to tuberculosis treatment.

MATERIALS AND METHODS

Drugs, reagents and chemicals

All drugs used in the study were procured from the Anti-Tuberculosis Clinic of the Obafemi Awolowo University Teaching Hospitals, Ile-Ife. All the reagents and chemicals used were of analytical grade and were obtained from Sigma, BDH or Randox Laboratories Limited, UK.

Plant

The dried seeds of *G. kola* were obtained from a local market in Ile-

lfe. They were identified and authenticated at the lfe Herbarium. The seeds were dehusked, chopped into pieces and oven dried at 40°C for 48 h and then ground to powder. The powdered material (120 g) was suspended in 1.2 L of 70% (v/v) ethanol for 72 h and filtered. The residue was extracted thrice using ethanol and followed by filtration. The combined filtrate was concentrated *in vacuo* at 45°C in a rotary evaporator to a semi-solid mass. The extract was suspended in normal saline and administered at different doses.

Animal treatment

Thirty adult albino rats (Wistar strain) of either sex (weights between 180 to 220 g) reared from the same colony were used for the experiment. They were maintained under standard laboratory conditions, fed with standard mouse chow (Ladokun Feeds, Ibadan, Nigeria) and provided with water *ad libitum*. The animals were randomly assigned into six groups (n = 5). Hepatotoxicity was induced using a combined dose of INH + RIF (7.5 mg/kg body weight of INH and 10 mg/kg body weight of RIF) administered intraperitoneally (i.p.). The following treatments were administered daily for 35 days:

- Group I (Control): Saline only (0.5 ml, i.p.).
- Group II: *Garcinia kola* only (60 mg/kg, p.o).
- Group III: INH + RIF only.
- Group IV: INH + RIF and *G. kola* seed extract (40 mg/kg, p.o).
- Group V: INH + RIF and *G. kola* seed extract (60 mg/kg, p.o).
- Group VI: INH + RIF and *G. kola* seed extract (80 mg/kg, p.o).

Sacrificing of animals

After 35 days of treatment, all animals were anaesthetized with diethyl ether and then dissected. Intra-cardiac blood samples were collected and plasma obtained by centrifugation (3000 rpm for 10 min). This was immediately stored in the refrigerator (4°C) and assayed within 48 h of collection. The whole liver was also excised upon dissection, and the wet weight obtained. A 10% (w/v) homogenate of each liver in chilled 250 mM-sucrose was immediately prepared with a Potter-Elvehjem homogenizer from a portion of the liver. The homogenates were centrifuged (5000 rpm for 10 min) and the supernatant immediately stored wet-frozen in the freezer (-20°C) and assayed within 48 h.

Estimation of alanine aminotransferase (ALT), aspartate aminotransferase (AST), alkaline phosphatase (ALP) and bilirubin levels in the plasma

ALP, ALT, AST and bilirubin levels in the plasma were assayed using assay kits and reagents utilizing principles based on methods as described by German Society for Clinical Chemistry (DGKC) (1972) (ALP), Reitman and Frankel (1957) (ALT and AST) and Jendrassik and Grof (1938) (bilirubin), all with slight modifications. Kits and reagents were made by Randox Laboratories Limited, UK.

Estimation of total protein in the plasma and liver

This was done using the Bradford method (Bradford, 1976) with slight modifications.

Estimation of glutathione (GSH), catalase (CAT), glutathione peroxidase (GPx), superoxide dismutase (SOD) and malondialdehyde (MDA) activity levels in the liver

Estimation of activity of oxidative stress biomarkers, GSH, CAT,

GPx, SOD and MDA were carried out according to methods as described by Moron et al. (1979), Sinha (1972), Rotruck et al. (1973), Misra and Fridovich (1972) and Parihar and Taruma (2003), respectively, all with slight modifications.

Histomorphological studies

Histomorphological investigation was performed by fixing the representative liver tissues from each group in 10% formal saline by total immersion for 48 h, after which they were processed via paraffin wax embedding according to standard methods (Drury and Wallington, 1980). The staining procedure of Haematoxylin and Eosin as described by Drury and Wallington (1980) was also adopted.

Statistical analysis

All values were expressed as mean \pm SEM (n = 5 in each group). One-way analysis of variance (ANOVA) was applied to test for significance of biochemical data of the different groups.

RESULTS

ALT activity levels in the plasma

ALT activity levels are as shown in Table 1. There was a higher level (up to 139%) of ALT in the group that was administered INH and RIF only (Group III) than the control group (Group I). ALT levels, however, decreased when *G. kola* was taken in combination with the antitubercular drugs. The difference was significant ($p < 0.05$) in those groups given dosages of 40 mg/kg (Group IV) and 60 mg/kg (Group V) bodyweight (bw) of *G. kola* seed extracts.

AST Activity Levels in the Plasma

As shown in Table 1, there was significantly ($p < 0.001$) lower levels of AST in the plasma of animals in the group administered the drug combination together with various doses of *G. kola* extract than in the group administered the drug combinations only.

ALP activity levels in the plasma

There were significant ($p < 0.001$) reduction of ALP level in the groups administered with the drug combination of INH and RIF, together with 40 mg/kg bw (45.43%), 60 mg/kg bw (67.96%) and 80 mg/kg bw (50.64%) doses of *G. kola* extract when compared with the group administered only the drug combination INH and RIF (116.328 \pm 13.336 U/l) as shown in Table 1.

Bilirubin concentration in the plasma

As is shown in Table 1, the group administered the anti-

Table 1. Effect of treatment with Isoniazid (INH) and Rifampicin (RIF) alone or with *Garcinia kola* seed extracts at various doses on liver antioxidant biomarkers, liver function biomarkers, and the total protein concentrations in the plasma and liver.

Groups parameter		I (Control)	II (<i>G. kola</i> only)	III ^a (INH + RIF)	IV ^b (INH + RIF + <i>G. kola</i> 40 mg/kg)	V ^b (INH + RIF + <i>G. kola</i> 60 mg/kg)	VI ^b (INH + RIF + <i>G. kola</i> 80 mg/kg)
Oxidative stress biomarkers	CAT (μmol of H_2O_2 consumed/min/mg protein)	15.83 \pm 0.05	27.50 \pm 2.78	11.53 \pm 2.57	28.38 \pm 1.12***	22.91 \pm 2.99*	19.53 \pm 1.41
	GPx (μmol /mg protein)	0.220 \pm 0.075	0.377 \pm 0.208	0.190 \pm 0.097	0.388 \pm 0.145	0.303 \pm 0.123	0.460 \pm 0.215
	SOD (units/mg protein)	1.570 \pm 0.188	1.157 \pm 0.523	0.568 \pm 0.245	2.830 \pm 0.496	1.812 \pm 0.305	1.466 \pm 1.071
	GSH (μmol /mg protein)	0.012 \pm 0.001	0.017 \pm 0.001	0.008 \pm 0.004	0.019 \pm 0.002	0.013 \pm 0.001	0.016 \pm 0.002
	MDA (μmol)	0.194 \pm 0.009	0.199 \pm 0.019	0.262 \pm 0.003	0.181 \pm 0.026	0.190 \pm 0.025	0.208 \pm 0.027
	Total protein (plasma; mg/ml)	8.328 \pm 0.891	7.612 \pm 0.521	6.587 \pm 0.462	7.900 \pm 0.627	7.938 \pm 0.246	8.325 \pm 0.542
	Total Protein (liver; mg/ml)	7.289 \pm 0.223	4.156 \pm 0.353	1.725 \pm 0.582***	4.133 \pm 0.157	5.274 \pm 0.781	6.050 \pm 0.362
Liver function biomarkers	ALT (U/L)	7.35 \pm 2.85	10.84 \pm 1.30	17.56 \pm 3.35	7.04 \pm 1.75*	6.40 \pm 3.09*	7.84 \pm 1.80
	ALP (U/L)	86.94 \pm 11.41	69.00 \pm 9.91	116.33 \pm 13.34	63.48 \pm 9.03*	37.26 \pm 12.77***	57.41 \pm 3.07**
	AST (U/L)	437.75 \pm 13.16	477.00 \pm 13.30	535.20 \pm 26.75	434.80 \pm 15.14**	362.00 \pm 20.94***	412.60 \pm 11.63***
	Bilirubin (mg/dl)	0.169 \pm 0.033	0.226 \pm 0.010	0.380 \pm 0.025***	0.139 \pm 0.023	0.127 \pm 0.009	0.125 \pm 0.025

Each value represents mean \pm SEM, n = 5. **p* value < 0.05, ***p* value < 0.01, ****p* value < 0.001. CAT = Catalase; SOD = Superoxide dismutase; GPx = Glutathione peroxidase; GSH = Glutathione; MDA = Malondialdehyde; ALT = Alanine aminotransferase; ALP = Alkaline phosphatase; AST = Aspartate aminotransferase; ^a= Group III was compared to Group I for statistical significance; ^b= Groups IV, V and VI were compared to Group III for statistical significance.

tubercular drugs alone, had a significantly (*p* < 0.001) higher bilirubin concentration than the control group. There were reductions in concentration of bilirubin in the groups after *G. kola* extracts were added with the drug combination.

Protein concentration in the plasma and liver

Protein concentration levels of the plasma and liver of the rats in the group administered the drug combination were lower by 20.91 and 76.33%, respectively, than in the control group. In both the plasma and the liver, the groups administered the drug combination together with *G. kola* seed extracts had higher protein levels than the group administered the drug combination only, with protein levels comparable to that of the control group in the rats in the group co-administered with 80 mg/kg bw *G. kola* seed extract.

GSH, CAT, GPx and SOD activities in the liver

GSH, CAT and SOD activities in the liver of the rats were highest in the groups administered the anti-tubercular drugs together with 40 mg/kg bw *G. kola* extract. Highest GPx activity was found in the group administered the anti-tubercular drugs in combination with 80 mg/kg dosage of *G. kola* seed extract. Lowest activity was found in the group that had only INH and RIF administered. Groups fed with various doses of *G. kola* extract had significantly higher levels of activity of the various enzyme biomarkers. This is illustrated in Table 1.

MDA levels in the liver

MDA levels, as shown in Table 1, were higher in the group administered only the anti-tubercular drugs INH and RIF than in every other group

including the control group and those administered with the antitubercular drugs together with doses of *G. kola* seed extracts.

Liver histomorphology

Images of the hepatocytes as observed under a microscope are shown in Plate 1. The hepatocytes of the rats of the control group showed a normal lobular architecture and normal hepatic cords of the liver. The hepatocytes are arranged in a regular pattern around the portal triad (portal vein, hepatic artery and bile duct) with sinusoids between excessive plates. Hepatocytes of the group administered only *G. kola* seed extracts also showed normal architecture, arranged normally around the portal triad. In the INH + RIF only treated group, the hepatocytes showed swelling, congestion and feathery degeneration. The arrangement of the hepatocytes in

this group was seen to be scattered, showing sign of degeneration, necrosis and vascular damage. The INH + RIF + *G. kola* (40 mg/kg) group showed hepatocyte congestion to some degree, but lobular architecture was more ordered on comparison with those of the control group. The hepatocytes of the INH + RIF + *G. kola* (60 mg/kg) group showed less congestion and more ordered lobular architecture than the INH + RIF + *G. kola* (40mg/kg) group, more similar to that of the control group. Hepatocytes of the INH + RIF + *G. kola* (80 mg/kg) group showed minimal changes with moderate portal triaditis, and their lobular architecture was normal. They showed tighter vascular congestion in the central vein, portal area and sinusoidal spaces than in the control group.

DISCUSSION

As can be deduced from Table 1, treatment with the antitubercular drugs alone elicited depression of CAT (27%), GPx (19%), SOD (63.80%) and GSH (33%) when compared to the control group. There was a concurrent elevation of MDA (35%) in the antitubercular drug group. These changes were reversed to different degrees by co-treatment with the different doses of *G. kola*. A consideration of the liver function biomarkers showed that there was significant elevation of ALT, ALP and AST in the INH + RIF group when compared to controls. As was observed with the oxidative stress biomarkers, co-treatment with *G. kola* lowered the levels of all these biomarkers. In similar manner, there was a very significant reduction in total protein in the liver in the INH + RIF group when compared to the control. As was observed with oxidative stress biomarkers, co-treatment with *G. kola* ameliorated this reduction.

Toxic metabolites are suggested to play a central role in the development of anti-tuberculosis drug-induced hepatotoxicity. Formation and detoxification of toxic metabolites can be affected by the inhibition, induction or genetic polymorphism in drug-metabolizing enzymes (Somasundaram et al., 2014). In this study, the ability of a 70% ethanol extract of *G. kola* seeds to protect against hepatotoxicity induced by anti-tubercular drugs, INH and RIF, was investigated. Metabolism of chemicals takes place largely in the liver, which accounts for the organ's susceptibility to metabolism-dependent, drug induced injury. The drug metabolites can be electrophilic chemicals or free radicals that undergo or promote a variety of chemical reactions, such as depletion of reduced glutathione; covalently binding to proteins, lipids or nucleic acids; or inducing lipid peroxidation (Kaplowitz, 2001). These destroy the integrity of the liver.

Lower level of activity, in the liver, of oxidative stress biomarkers (GSH, SOD, CAT and GPx) in the group administered INH and RIF only compared to the control group is indicative of an inhibition of these enzymes by

toxic metabolites of the drugs. This makes them unable to scavenge and mop up free radicals generated by the anti-TB drugs used. Higher levels in the groups co-administered with various doses of *G. kola* extract indicates the protective effect of the *G. kola* extract, either by inducing the elevation of activities of these enzymes or its active principles also acting as free radical scavengers, thus overwhelming the inhibition activity of the anti-tubercular drug metabolites.

Liver enzyme biomarkers are enzymes specialized and concentrated mainly in the liver which upon injury to the liver, seep into other bodily fluids. A measure of their levels in those bodily fluids is an indication of the extent of liver damage (if any). Examples of these hepatic biomarkers are ALP, AST, and in particular ALT.

In this study, hepatotoxicity in Wistar rats was produced by the administration of the anti-tubercular drug combination INH (7.5 mg/kg) and RIF (10 mg/kg) every day for 35 days. This is indicated by the higher plasma levels of ALP, AST and ALT on comparison with the control group. Mitigation of the toxicity of the anti-tubercular drugs is affected by co-administration with various doses of ethanol extract of *G. kola* seeds. This is indicated by the lower levels of ALT, ALP and AST in the group of animals administered the drugs together with doses of *G. kola* seed extracts Table 1. Bilirubin concentration was significantly ($p < 0.001$) higher in the INH-RIF only treated group. This is due to the inability of the liver to conjugate bilirubin with glucuronide, thereby generating an accumulation of unconjugated bilirubin in the blood. Co-administration of *G. kola* seed extracts with the anti-tubercular drugs mitigated hepatic damage, hence improved bilirubin-glucuronide conjugation in the liver.

Total protein in both the plasma and liver were seen to be lowered in the INH-RIF only treated group. This is as a result of the inability of the liver to synthesize proteins. In the INH-RIF and *G. kola* seed extract treated groups, the *G. kola* extract mitigated the damage done by the anti-tubercular drugs, thus, resulting in higher protein level in the plasma and liver. A particularly important consequence of free radical damage in many cells is the peroxidation of lipids in the membranes, which results in the formation of lipid peroxides and aldehydes. MDA is one of the major products of peroxidation in cells and is usually quantitatively measured by its reaction with thiobarbituric acid to measure level of oxidation. MDA levels were observed to be higher in the INH-RIF only treated group than the control group and the groups co-administration with anti-tubercular drugs and various doses of ethanol extract of *G. kola* seeds. This higher level could be as a result of damage imposed by the increased free radicals generated during the detoxification of the drugs. The resulting lower levels of MDA in the groups co-administered with anti-tubercular drugs and the *G. kola* seed extracts indicate a lower level of peroxidation in the liver.

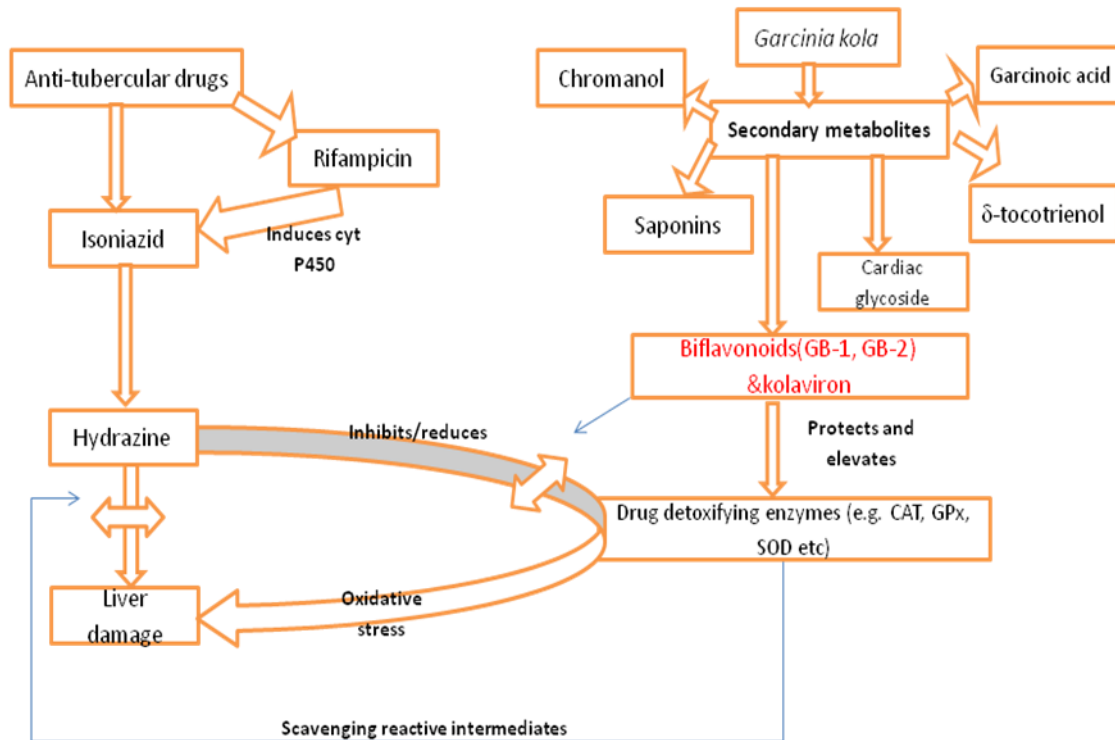


Figure 1. Possible actions of *G. kola* as a hepatoprotective agent.

The Rifampicin induces Cytochrome P450 which is a drug metabolizing enzyme. By inducing this enzyme, it acts by metabolizing more of the INH, thereby producing more toxic hydrazine (metabolite of INH). These hydrazines act by inhibiting or reducing the drug detoxifying enzymes, thereby making the detoxifying enzymes unable to scavenge these free radicals been produced, causing oxidative stress which eventually leads to liver toxicity or liver damage (Figure 1). Studies indicate that there is no clear evidence that INH proves much more injuries than RIF and in this connection, they consider that it is the combination of these two drugs that confers the additive or even synergistic potential of liver toxicity than either agent alone, as conjectured. INH is metabolized in the liver primarily by acetylation and hydrolysis, and it is these acetylated metabolites that are thought to be hepatotoxic. Reports on rats suggest that the hydrazine metabolite of INH which has subsequent effect on CYP2E1 induction is involved in the development of INH-induced hepatotoxicity, and also oxidative stress as one of the mechanism for INH + RIF induced hepatic injury (Saleem et al., 2008; Yue et al., 2004).

G. kola with its chemical constituent such as the kolaviron is believed to protect and elevate the activities of the drug detoxifying enzymes, thereby protecting the liver against liver damage (Figure 1). The varied chemical composition of *G. kola* seed extract makes it difficult to

assign its hepatoprotective property to any one of its constituent chemicals. Kolaviron has been reported to significantly prevent hepatotoxicity induced by several hepatotoxic agents such as phalloidin, thioacetamide and paracetamol (Iwu et al., 1987; Akintowa and Essien, 1990). The hepatoprotective property of *G. kola* seed extract may also be because of its other properties like anti-inflammatory property which may prevent inflammatory hepatic damage, immune-modulating property and antioxidant property thereby reducing the oxidative stress imposed by the drugs; this antioxidant mechanism seems to be important as *G. kola* extract has been shown to reduce oxidative stress and oxidative stress has been found to be the most important mechanism in hepatotoxicity of anti-tubercular drugs (Iwu et al., 1987; Akintowa and Essien, 1990).

To examine the extent of hepatic damage, by the anti-tubercular drugs, and mitigation, by the *G. kola* seed extract, histomorphological examinations were carried out. Degeneration of cells, vascular congestion and necrosis were observed in the INH-RIF only treated group (Plate 1C). Varying levels of recovery of the cells were seen in the INH-RIF and *G. kola* treated groups at different concentrations of the *G. kola* seed extract. However, at 40 mg/kg bw *G. kola* extract co-administered with INH-RIF, the hepatocytes' arrangement is closer to normal than the INH-RIF only treated group, with a lesser degree of congestion (Plate 1D). The 60 mg/kg bw *G.*

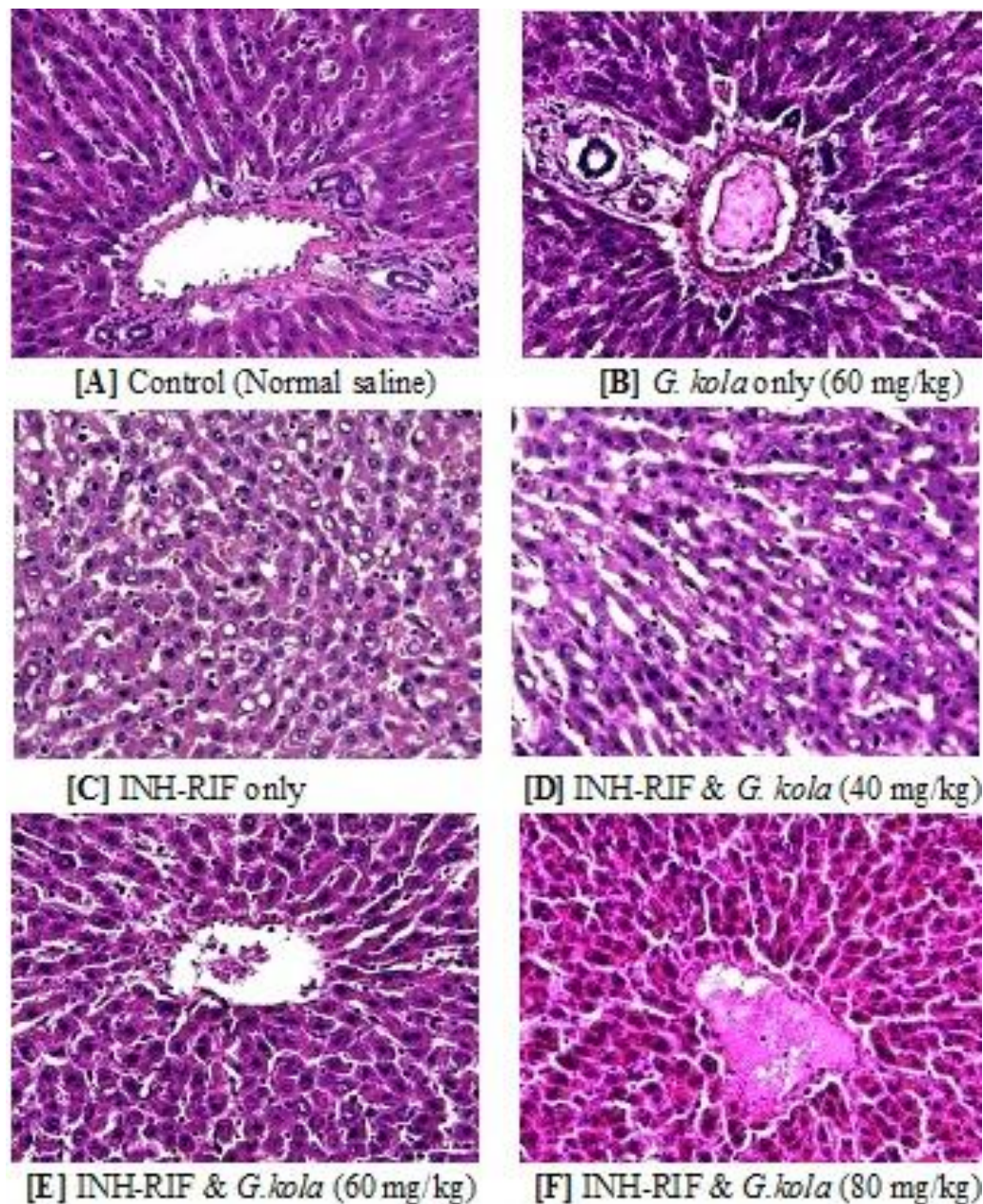


Plate 1. Liver histomorphology of animals treated with isoniazid (INH), rifampicin (RIF) and *G. kola* seed extracts (H&E $\times 400$).

kola group's hepatocytes showed very minor congestion and close to normal lobular architecture (Plate 1E). The hepatocytes of the 80 mg/kg bw *G. kola* group had normal cellular arrangement, however, the tighter vascular congestions in the central vein, portal area and sinusoidal spaces indicate a higher concentration of the *G. kola* extract and could not protect the liver significantly by recovering from the damage done by the anti-TB drugs. Plate 1D, E and F showed the potency of the *G. kola* extract in protecting the liver against serious damage by the anti-TB drugs induce hepatotoxicity.

Conclusion

This study demonstrates the hepatoprotective effect of *G. kola* seed extracts when co-administered with anti-tubercular drugs. Optimum mitigation was achieved at the 60 mg/kg bw dosage of the ethanol extracts of the *G. kola* seeds. This hepatoprotective effect could be due to its high content of flavonoids. Although this study was done with rats, the findings offer a way of combating the harmful side-effects of antitubercular treatment. The study clearly highlights the possibility of mitigating

hepatotoxicity of antituberculosis drugs with a simple extract from *G. kola*, a readily accessible plant. After larger studies and further fractionation to identify the protective compounds, its co-administration could make treatment easier for the patients. This in turn would improve patient compliance and reduce the risk of developing resistance or spreading the disease.

Conflict of interest

Authors declare that there are no conflicts of interests

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