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Antimicrobial activity and DNA-fragmentation effect of isoflavonoids isolated from seeds of *Millettia ferruginea*, an endemic legume tree in Ethiopia

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ABSTRACT

Objective: To investigate the seeds of *Millettia ferruginea* (*M. ferruginea*) to unravel its antibacterial, antifungal, antitubercular, and antileishmanial potential for the first time.
Methods: *M. ferruginea* seeds were refluxed separately with chloroform, methanol and water to prepare the three extracts, which were tested against the reference strains of Gram-positive and Gram-negative bacteria, yeast cells, *Mycobacterium tuberculosis* and *Leishmania donovani* (*L. donovani*) promastigotes. Next, the seeds were chemically analysed to isolate three constituent compounds, *viz.*, barbigerone, calopogonium isoflavone-A and durmillone, which were purified, characterised and evaluated for antibacterial and antileishmanial activity. Further, Comet assay was conducted to observe DNA fragmentation effects on human peripheral blood mononuclear cells pretreated with the isoflavonoid compounds.
Results: The chloroform and methanol extracts of *M. ferruginea* seeds exhibited antibacterial and antileishmanial activity. The pure compounds also showed inhibitory activity against Gram-negative ATCC strains (minimum inhibitory concentration ~0.5 μmol/L), and *L. donovani* promastigotes (IC_{50} 8.2–87.3 μg/mL). However, they had little or no activity against yeast cells and tubercle bacilli. The DNA fragmentation study showed that the isoflavonoid constituents of *M. ferruginea* seeds were safe at therapeutic doses.
Conclusions: The antibacterial efficacy of the non-aqueous extracts of *M. ferruginea* seed was observed against both Gram-positive and Gram-negative ATCC strains. Moreover, the constituents isoflavonoids, *viz.*, barbigerone, calopogonium isoflavone-A and durmillone, exhibited inhibitory activity against Gram-negative ATCC strains and *L. donovani* promastigotes. The comet assay showed that the compounds were safe to be considered for human consumption.

1. Introduction

Millettia ferruginea (Hochst.) Baker (*M. ferruginea*) plants, with two distinct subspecies, namely, ferruginea and darassana belonging to Fabaceae (Leguminosae) family, are widely distributed within the agro-climatic zones of 1 000–2 500 m above the sea level in Ethiopia[1]. The leguminous *M. ferruginea* trees would grow even up to 25 m high and often found in association with essential crops, like barley, sorghum and maize, demonstrating a beneficial effect owing to its nitrogen-fixing property. It also serves as the shade tree in plantation, and feeds the honeybees on its flowers, while the leaves and shoots are mostly taken as fodder for the ruminants. Therefore, this endemic plant, known as "berbera" or "brebra" in local Amharic language, plays an important role for the regional agro-forestry in Ethiopia[2]. In fact, a recent study showed that "brebra" seed flour is exceptionally rich in protein and also safe in terms of cyanide and other antinutritional content. The authors have highlighted the economic prospect of *M. ferruginea* seeds for extraction of the oil as biodiesel, and to make soaps, followed by utilisation of the byproduct oil-cake as proteinaceous food for both humans and livestock[2].

Phytochemical analysis of *M. ferruginea* was undertaken by several workers revealing a number of rotenoids, flavonoids and

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chalcones isolated from the seeds and bark[3-5]. However, only a limited study towards scientific validation of folkloric application of this plant, mainly for insecticidal purpose, has been reported so far. The common practice to stupefy and catch fish with powdered seeds of *M. ferruginea* was attributed to rotenone and analogous piscicidal compounds found in the legumes[3]. Several studies were conducted to corroborate the traditional usage of the seed extracts for controlling some storage pests, such as adzuki bean beetle, maize weevil and bean bruchid, as well as mosquito larvae[6-8]. Thus, Tilahun and Azerefegne (2013), confirmed that the aqueous extract of *M. ferruginea* seeds was toxic against the larvae of a stem borer insect (*Busseola fusca*), and supported its application for pest management in maize plantations[9]. Again, the acaricidal activity of the seeds was demonstrated against the tropical bont tick by Choudhury *et al*. (2015)[10]. However, the medicinal application of *M. ferruginea* is rather limited to its wound-healing property, as the local farmers use the seeds for dressing the typical skin infection ('mujele') caused in their feet by a soil insect[11]. Truly, this endemic plant deserves more attention not only due to the prospective socio-economic contribution to Ethiopia, but also in view of the abundant content of pyrano- and C-prenylated isoflavones with potential pharmacological activity[12-15]. Recently, phytochemical studies have indicated the antiparasitic property of the compounds isolated from *Millettia* plants against vector-borne diseases like malaria and leishmaniasis[16,17].

Presently, we carried out a systematic investigation on the antibacterial, antifungal, antitubercular and antileishmanial activity of *M. ferruginea* seed extracts. Also, we have isolated and characterised three isoflavonoids, namely, barbigerone, calopogonium isoflavone-A and durmillone from the mature seeds in order to corroborate the antimicrobial activity of *M. ferruginea* for the first time. In addition, comet assay on human peripheral blood mononuclear cells (PBMCs) was carried out to evaluate the possibility of DNA-damaging effect of these isoflavonoid constituents found in several species of *Millettia* plants found in Africa. This study was undertaken in view of the reported acaricidal and piscicidal activity of *M. ferruginea* seeds *vis-à-vis* the unexplored potential for development of *M. ferruginea* seeds as an endemic resource of Ethiopia[1,10]. This is the first such study undertaken on the three isoflavonoids to the best of our knowledge.

2. Materials and methods

2.1. Plant material

The mature seeds of *M. ferruginea* subspecies darassana were collected from a place near Dilla University campus at Dilla, and duly authenticated at the National Herbarium, Addis Ababa. A voucher specimen (S-108) of the plant sample was deposited at the Department of Biology, Addis Ababa University, Addis Ababa, Ethiopia. Dried seeds (100 g) were ground by using a mortar and pestle. Three portions of the powdered sample (10 g of each) were refluxed separately in 100 mL each of chloroform, methanol and water for 2 h to obtain the respective extracts. After filtration, the solvents (chloroform/methanol) were removed in a rotary evaporator, and the water extract was freeze-dried in a lyophiliser. The yield (% w/w) of each of the crude extracts of *M. ferruginea* was obtained from chloroform, methanol and water.

2.2. Isolation and characterisation of compounds

The reagents and solvents for column chromatography were procured from E Merck, India. All solvents were distilled before use. Thin-layer chromatography of the products was performed using silica gel G and petroleum ether (60–80 ˚C): ethyl acetate (7:3), and the spots were visualised by keeping the glass plates in UV and iodine chamber. UV (in MeOH) and infrared radiation (IR) spectra (in KBr) were recorded on a Shimadzu UV-1800 spectrophotometer and Shimadzu IR Affinity-1 spectrophotometer respectively. ^{1}H nuclear magnetic resonance (NMR) in $CDCl_3$ was recorded in a Bruker BioSpin AG 400 MHz NMR spectrometer, using tetramethylsilane as an internal standard.

The powdered seeds (100 g) were extracted at room temperature by maceration with hexane (600 mL) for a week. The solvent was completely removed in a rotary evaporator to obtain a residue of thick oil with a pale yellow colour (~33 g). After keeping the oil at 4 ˚C for one week, a white precipitate was obtained, which was filtered under cold condition. The residue was washed with cold hexane, and crystallized from methanol to afford durmillone (35 mg); R_f = 0.48, melting point 181–182 ˚C.

After extraction with hexane, the remaining solid residue (~67 g) was further extracted in a Soxhlet apparatus, with dichloromethane (250 mL) for 8 h. The solvent was removed under reduced pressure to obtain a deep brown sticky solid (7.3 g). This was subjected to column chromatography (column size: length 280 mm, diameter 15 mm) over silica gel (60–120 meshes, 75 g), and eluted by gradual increase in polarity of the solvent mixture of petroleum ether and ethyl acetate. Fractions 11–19 (5% ethyl acetate) afforded a white crystalline solid, which was crystallised from petroleum ether-chloroform followed by methanol to get a crystalline compound characterized as calopogonium isoflavone-A (70 mg, 0.07%); R_f = 0.70, melting point 137–139 ˚C. Fractions 36–48 (10% ethyl acetate) again afforded a good amount of durmillone (~210 mg, 0.24%) after crystallisation from methanol. Finally, the fractions 55–58 (15% ethyl acetate) on crystallisation from petroleum ether-ethyl acetate yielded cream coloured compound were characterized as barbigerone (40 mg, 0.04%); R_f = 0.30, melting point 150–151 ˚C. Spectroscopic analysis of UV, IR and ^{1}H-NMR and mass spectrometer (MS) was carried out for characterisation of the three purified isoflavonoids followed by comparison with the available data from earlier literature.

2.3. Test procedures

2.3.1. Antibacterial activity

Extracts (chloroform/methanol/water) of *M. ferruginea* were dissolved in dimethyl sulfoxide (DMSO) (10 mg/mL), and tested for antibacterial activity by the agar well diffusion assay against both Gram-positive and Gram-negative bacteria from American Type Culture Collection[18]. The bacterial culture in Muller-Hinton broth was adjusted to the final inoculum density of 1×10^7 CFU/mL (by 0.5 McFarland standard, HiMedia, Mumbai, India) on molten

Mueller-Hinton agar plates. After solidification, wells (diameter 9 mm) were made with a sterile borer in the inoculated Mueller-Hinton agar plates. About 100 μL solution containing 1 mg of each extract was dispensed in the wells. Similarly, the isoflavonoids (barbigerone, calopogonium isoflavone-A and durmillone) were also tested by dispensing 100 μL solution per well in varying concentrations (0.1, 0.5, 1.0, 5.0, 25.0, 100.0 μmol/L) of each compounds. DMSO was tested as the vehicle control, while penicillin G, streptomycin and gentamicin were the standard drugs used as positive controls in this assay. Antibacterial activity was expressed as the diameter of inhibition zone produced around each well by the plant extract/compound/antibiotic, and was measured after 24 h of incubation at 37 ℃. The minimum quantity of the tested extracts/compounds that could produce visible zone of inhibition in Mueller-Hinton agar plate was considered as the minimum inhibitory concentration (MIC) of the corresponding extracts/compounds. Each test was conducted in triplicate to confirm the reproducibility of the observed data.

2.3.2. Antifungal activity

The plant extracts and compounds were tested to determine MIC for antifungal activity against two ATCC strains of *Candida albicans* (ATCC 10231) (*C. albicans*) and *Cryptococcus neoformans* (ATCC 66031) (*C. neoformans*) by broth micro-dilution procedure[18]. Stock solutions (2.048 mg/mL) of chloroform/methanol/water of *M. ferruginea* and the three isoflavonoid constituents were prepared in DMSO, and serially diluted in Sabouraud dextrose broth in a concentration ranged from 0.5 to 1 024 μg/mL in a 96-well microtitre plate. The broth culture containing 0.5 McFarland (1×10^8 CFU/mL) inoculum density was then introduced to each of the microtitre wells at 1:10 ratio to maintain final inoculum density of 1×10^7 CFU/mL. In addition, one row of 12 wells contained only Sabouraud dextrose broth (100 μL) as a 'blank', while another row contained only DMSO as the 'vehicle control' serially diluted with Sabouraud dextrose broth (v/v) to the concentration corresponding to the respective wells used for MIC determination of extracts/compounds. The culture plates were incubated at 35 ℃ for 48 h and the presence of visible growth in each well was inferred by measuring optical density at 630 nm using ELISA reader (Erba LisaScan II, Transasia). The minimum concentration of the extracts/compounds that did not exhibit visible fungal growth was recorded as the MIC of the corresponding extracts/compounds.

To determine minimum fungicidal concentration, a loop-full of culture was taken from each well containing different dilutions of the tested extracts/compounds and inoculated by a 4 mm loop (calibrated to 0.01 mL) on Sabouraud dextrose agar plate. The lowest concentrations of the tested extracts/compounds that did not support the growth of visible fungal colony were noted as the minimum fungicidal concentration of the respective extract/compound. Two antifungal drugs, *viz.*, amphotericin B and nystatin were used as the positive controls in this assay.

2.3.3. Antitubercular activity

Tetrazolium microplate assay with 3-(4, 5-dimethyl thiazol-2-yl)-2, 5-diphenyl-tetrazolium bromide (MTT, Sigma-Aldrich, Saint Louis, USA) was performed to test the antitubercular activity of the extracts (chloroform/methanol/water) of *M. ferruginea*, and one of the isoflavonoids (durmillone) against *Mycobacterium tuberculosis* H_{37}Ra strain (ATCC 25177) (*M. tuberculosis*) using the standard protocol[19]. The tested range of concentration for the extracts and durmillone was 1024–0.5 μg/mL. The MIC was the lowest concentration of the tested sample at which no tubercular growth was indicated by the colour change of MTT. To determine the minimum bactericidal concentration, a loop-full of culture from each well containing different dilutions of the tested samples was streaked on solid Löwenstein-Jensen medium (HiMedia, Mumbai, India). The lowest concentration of the tested extracts/durmillone that did not support the growth of visible mycobacterial colony on Löwenstein-Jensen medium was recorded as the minimum bactericidal concentration[20].

2.3.4. Antileishmanial activity

The crude extracts and purified isoflavones obtained from *M. ferruginea* seeds were tested for antileishmanial activity by performing colorimetric MTT assay as described previously[21]. Briefly, *Leishmania donovani* (AG 83) (*L. donovani*) promastigotes (5×10^5 cells/mL; 300 μL) were incubated at (22 ± 2) ℃ after treatment with and without the samples tested at the concentration range of 10–500 μg/mL for the extracts, and 1–100 μmol/L for the isoflavones. Amphotericin B was tested as a positive control at 0.01–10.00 μmol/L. The cells were harvested after 72 h, resuspended in poly butylenes succinate (PBS) (500 μL) containing MTT (0.3 mg/mL) and kept in darkness for about 18 h. The dehydrogenase enzyme presenting in the living cells of *L. donovani* promastigotes would reduce the yellow coloured MTT into purple formazan crystals, which were dissolved in DMSO for measurement of the optical density at 570 nm (Bio-Rad ELISA reader, model 680, Hercules, USA). Hence, the percentage of cell growth was calculated by comparing the optical density of the test samples with the vehicle control (100%) in order to estimate the IC_{50} value as a measure of the inhibitory activity of the tested plant products determined from a dose-response curve drawn by Origin 5.0 software (Microcal Software, Inc., Northampton, USA).

2.3.5. Evaluation of DNA damage by comet assay

DNA fragmentation activity of the isoflavonoids (barbigerone, calopogonium isoflavone-A and durmillone) was evaluated against human PBMCs.

2.3.5.1. Isolation of PBMCs

PBMCs were isolated from the ethylene diamine tetraacetic acid blood samples of healthy and non-smoking in-house donors registered at Ashok Laboratory Centre for Transfusion Medicine and Clinical Research (Blood Bank), keeping the confidentiality intact. PBMCs were separated by ficoll (Histopaque® 1077, Sigma-Aldrich, Saint Louis, USA) density gradient centrifugation, followed by repeated washing with ice-cold PBS (pH 7.4). The yield of lymphocytes was determined by automated cell counter (Sysmex XT-1800i, Kobe, Japan), and resuspended in cold PBS to a final concentration of ~2×10^6 cells/mL.

2.3.5.2. Comet assay

The compounds were dissolved in DMSO to prepare stock solutions (10 mmol/L) for the assay. The PBMC suspension was diluted (2×)

with suitable concentration of the respective test solution in order to obtain the final treatment doses (1 μmol/L and 1 mmol/L), and the reaction mixtures were incubated for 1 h at 37 ˚C. Another set of PBMC treated with DMSO (10 μL taken in 1 mL of nuclease-free deionized water) was served as negative control, while cells treated with H_2O_2 (100 μmol/L) were taken as positive control for this assay. Moreover, intertest variability was avoided by performing all the analyses at the same time point.

Aliquots from PBMC suspension treated with barbigerone/ calopogonium isoflavone-A/durmillone, along with the negative and positive controls, were further diluted in PBS (Ca^{2+} and Mg^{2+} free; pH 7.4) at a ratio of 1:10 (v/v) to attain final cell density of ~1 × 10^5 CFU/mL. Alkaline single cell gel electrophoresis (or comet assay) was performed by CometAssay® reagent kit (catalog # 4250-050-K, Trevigen, Gaithersburg, US) according to the manufacturer's instructions. Briefly, PBMC suspension (~1 × 10^5 cells/mL) in PBS was combined with 1% molten low-melting agarose (Catalog # 4250-050-02) at 37 ˚C at a ratio of 1:10 (v/v), and transferred to the comet slide (Catalog # 4250-050-03). After gelling time (~30 min), the slide was immersed in pre-chilled (4 ˚C) lysis solution (Catalog # 4250-050-01) and alkaline solution (200 mmol/L NaOH, 1 mmol/L ethylene diamine tetraacetic acid, pH > 13) respectively for 45 min in each. Then, electrophoresis of the slides was conducted in pre-chilled (4 ˚C) alkaline electrophoresis solution (200 mmol/L NaOH, 1 mmol/L ethylene diamine tetraacetic acid, pH > 13) for 30 min at 25 v and 300 mA (1.5 v/cm), and then washed in deionized water, dehydrated in 70% ethanol and stained with SYBR® Green I (Catalog # 4250-050-05, maximum excitation/emission was 494 nm/521 nm) diluted in tris-edta buffer (10 mmol/L Tris-HCl pH 7.5, 1 mmol/L ethylene diamine tetraacetic acid). Fluorescent comet patterns were examined with a Motic BA400 microscope under 200× magnification and fluorescein isothiocyanate filter combination. The resultant images were captured by a charge-couple device camera[22].

2.3.5.3. Image analysis

Comet score v1.5 (Tritek Corporation) was used to measure the percentage of DNA in head (comet head intensity divided by the total comet intensity, multiplied by 100), tail length (comet head diameter subtracted from the comet length), the percentage of DNA in tail (comet tail intensity divided by the total comet intensity, multiplied by 100), and tail moment (% of DNA in the comet tail multiplied by the tail length). Hundred cells per sample were observed by this software. Results were compared with negative control, using Student's *t*-test, and differences were considered statistically significant at $P < 0.05$, and highly significant at $P < 0.01$ and $P < 0.001$.

3. Results

3.1. Isolation and characterisation of isoflavonoid compounds from M. ferruginea seeds

The three compounds isolated in our study were characterised as calopogonium isoflavone-A, durmillone and barbigerone through meticulous spectroscopic analysis (as presented below), and the chemical structures (Figure 1) were confirmed by comparison with respective data obtained from the literature[4,5].

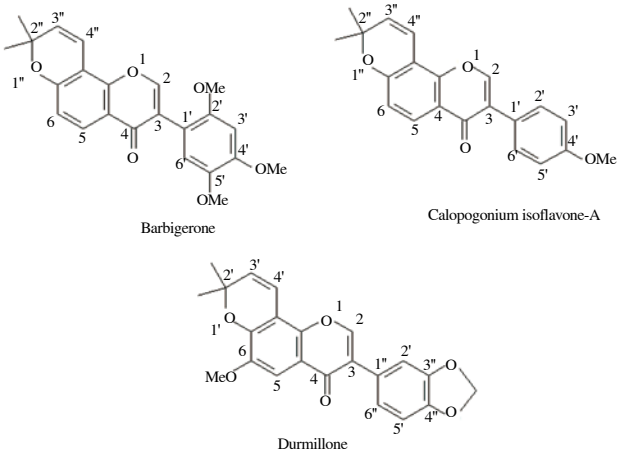

Figure 1. Chemical structures of isoflavonoids isolated from *M. ferruginea* seeds.

Calopogonium isoflavone-A: UV λ_{max} nm (log ε): 262 (4.6380), 312 (3.8010), 322 (3.8126). IR (KBr, cm^{-1}): 1642 (C = O), 1632, 1608 (aromatic), 1570, 1392, 1380 [$(CH_3)_2C$], 1282, 1040, 1032(OCH_3), 885, 831, 761. ^{1}H-NMR (δ): 1.50 (6H, s, $2CH_3$, gem dimethyl, C-2"), 3.84 (3H, s, OCH_3, C-4'), 7.50 (1H, d, *J* = 9 Hz, H-2'/H-6', ortho coupling with H-3'/H-5'), 6.86 (1H, d, *J* = 9 Hz, H-3'/H-5', ortho coupling with H-2'/H-6'), 5.72 (1H, d, *J* = 10 Hz, H-4", olefinic cis-coupling with H-3"), 6.81 (1H, d, *J* = 10 Hz, H-3", olefinic cis-coupling with H-4"), 6.97 (1H, d, *J* = 8.7 Hz, H-6, ortho coupling with H-5), 8.06 (1H, d, *J* = 8.7 Hz, H-5, ortho coupling with H-6), 7.94 (1H, s, H-2). MS (EI); *m/z* 334 (M^+), $C_{21}H_{18}O_4$.

Durmillone: UV λ_{max} nm (log ε): 229 (4.3780), 262 (4.4124), 333 (3.9191), 346 (3.8771). IR: (KBr, cm^{-1}): 1630 (C = O), 1588 (aromatic), 1467, 1394, 1380 [$(CH_3)_2C$], 1275, 1241, 1036, 929, 898. ^{1}H-NMR (δ): 1.50 (6H, s, $2CH_3$, gem dimethyl, C-2"), 3.96 (3H, s, OCH_3, C-6), 5.97 (2H, s, -O-CH_2-O-, C-3'/C-4'), 5.75 (1H, d, *J* = 10 Hz, H-4", olefinic cis-coupling with H-3"), 6.81 (1H, d, *J* = 10 Hz, H-3", olefinic cis-coupling with H-4"). 6.87 (1H, d, *J* = 8 Hz, H-5', ortho coupling with H-6'), 6.97 (1H, d, *J* = 8 Hz, H-6', ortho-coupling with H-5'), 7.11 (1H, s, H-2'), 7.57 (1H, s, H-5), 7.94 (1H, s, H-2). MS (EI); *m/z* 378 (M^+), $C_{22}H_{18}O_6$. The data were comparable with published literature[4,5].

Barbigerone: UV λ_{max} nm (log ε): 232 (4.6027), 259 (4.6489), 262 (4.6509), 297(4.2067), 325 (sh). IR (KBr, cm^{-1}): 1641 (C = O), 1627, 1599 (aromatic), 1468, 1440, 1394, 1382, 1313, 1278, 1215, 1209, 1032 (OCH_3), 808, 776, 767. ^{1}H-NMR (δ): 1.50 (6H, s, $2CH_3$, gem dimethyl, C-2"), 3.93 (3H, s, OCH_3, H-2'), 3.86 (3H, s, OCH_3, H-4'), 3.78 (3H, s, OCH_3, H-5'), 6.63 (1H, s, H-3'), 6.95 (1H, s, H-6'), 5.72 (1H, d, *J* = 10 Hz, H-4", olefinic cis-coupling with H-3"), 6.82 (1H, d, *J* = 10 Hz, H-3", olefinic cis-coupling with H-4"), 6.80 (1H, d, *J* = 9 Hz, H-6, ortho coupling with H-5), 8.05 (1H, d, *J* = 9 Hz, H-5, ortho coupling with H-6), 7.93 (1H, s, H-2). MS (EI); *m/z* 394 (M^+), $C_{23}H_{22}O_6$.

3.2. Antibacterial activity

Antibacterial activity of *M. ferruginea* seed extracts (chloroform/ methanol/water) were evaluated against ATCC strains, both Gram-positive and Gram-negative. The zone of inhibition exhibited in the presence of each of the extracts and three standard antibiotics were determined by agar well diffusion assay. The data presented in Table

1 showed that the chloroform and methanol extracts of *M. ferruginea* were moderately active against the tested bacteria, methanol extract of *M. ferruginea* being more active than chloroform extract of *M. ferruginea*. In fact, the activity of the methanolic extract against *Pseudomonas aeruginosa* (ATCC 27853) (*P. aeruginosa*) was comparable with a positive control (streptomycin). However, the water extract of *M. ferruginea* extract was found to be ineffective against all the tested bacteria.

Table 1
Antibacterial activity of *M. ferruginea* seed extracts.

Bacteria (source)		Inhibition zones of bacterial growth (mm*)					
		Extracts (1 mg/well)			Standard antibiotics		
		C	M	W	P	S	G
GPB	*Staphylococcus aureus* (ATCC 25922)	12	13	–	26	25	16
	Enterococcus faecalis (ATCC 29212)	11	14	–	19	17	ND
GNB	*E. coli* (ATCC 35218)	11	14	–	ND	15	ND
	P. aeruginosa (ATCC 27853)	14	15	–	ND	15	18

GPB: Gram-positive bacteria; GNB: Gram-negative bacteria; *: Including diameter of well (9 mm); –: No activity; ND: Not determined; C: Chloroform; M: Methanolic; W: Water. *E. coli* (ATCC 35218): *Escherichia coli*; P: Penicillin G (0.001 mg/well); S: Streptomycin (0.01 mg/well); G: Gentamicin (0.01 mg/well).

In view of the positive findings, three isoflavonoid constituents of the *M. ferruginea* seed extracts, *viz.*, barbigerone, calopogonium isoflavone-A and durmillone, were tested against four clinically relevant ATCC strains of Gram-negative bacteria. Table 2 showed agar-well diffusion data of individual compounds and a positive control (streptomycin) against these strains. All three compounds exhibited MIC of 0.5 μmol/L against the ATCC strains of *E. coli*, *Klebsiella pneumoniae* and *P. aeruginosa*. In case of *E. coli*, zone diameters in the range of 17–20 mm were produced by the compounds applied at comparatively lower dose (5 μmol/L) than streptomycin (18 mm; ~0.2 mmol/L). However, durmillone showed no activity against *Proteus vulgaris* (ATCC 6896).

3.3. Antifungal activity

M. ferruginea seed extracts and isolated compounds were tested against the ATCC strains of *C. albicans* and *C. neoformans* by broth microdilution assay. Two antifungal drugs were tested as positive controls. Table 3 showed that the crude extracts exhibited moderate activity against the yeasts at a MIC range of 128–512 μg/mL. However, MIC values of barbigerone and calopogonium isoflavone-A (256 μg/mL) indicated weak antifungal activity, while durmillone did not respond at the highest tested dose of 1 024 μg/mL.

3.4. Antitubercular activity

Antitubercular activity of the three extracts and durmillone were evaluated against a reference strain of *M. tuberculosis* $H_{37}Ra$ by tetrazolium microplate assay (Table 4). Chloroform and methanol extracts of *M. ferruginea* did not exhibit antitubercular efficacy (MIC = 2 048 μg/mL), although the water extract was found to be comparatively more active at MIC of 512 μg/mL. Out of the three isolated compounds, only durmillone was tested, and the MIC was found at 512 μg/mL (~1.4 mmol/L).

Table 4
MIC and minimum bactericidal concentration of the crude extracts and durmillone against *M. tuberculosis* $H_{37}Ra$.

Bacterium	Activity (μg/mL)	Extracts			Durmillone	Ciprofloxacin*
		Chloroform	Methanolic	Water		
MT	MIC	2 048	2 048	512	512	2
	MBC	> 2 048	> 2 048	1 024	512	2

MT: *M. tuberculosis* $H_{37}Ra$ (ATCC 25177); *: Ciprofloxacin was used as a positive control. MBC: minimum bactericidal concentration.

3.5. Antileishmanial activity

M. ferruginea seed extracts were evaluated for antileishmanial activity by MTT reduction assay on *L. donovani* promastigotes. The data in Table 5 showed that water and chloroform extracts of *M. ferruginea* could effectively inhibit the cell growth at IC_{50} values of 38 and 183 μg/mL respectively. Methanol extract of *M. ferruginea* was more active than chloroform extract of *M. ferruginea* exhibiting ~5-fold greater activity. In contrast, water extract of *M. ferruginea* did not inhibit cell growth at the highest tested dose of 500 μg/mL.

Table 2
Antibacterial activity (inhibition zone in mm[a]) of barbigerone, calopogonium isoflavone-A and durmillone evaluated against Gram-negative strains by agar-well diffusion method.

Bacteria	Barbigerone (μmol/L)					Calopogonium isoflavone-A (μmol/L)					Durmillone (μmol/L)					Streptomycin* (mg/well)	MIC# (μmol/L)		
	0.1	0.5	1.0	5.0	25.0	0.1	0.5	1.0	5.0	25.0	0.1	0.5	1.0	5.0	25.0	0.01	B	C	D
EC	–	14	15	19	20	–	13	18	20	20	–	12	15	17	17	18	0.5	0.5	0.5
KP	12	12	18	17	19	–	10	12	12	14	–	11	12	14	15	21	< 0.1	0.5	0.5
PA	–	11	12	13	13	–	11	12	11	12	–	11	12	13	15	12	0.5	0.5	0.5
PV	–	–	12	13	13	–	12	12	12	13	–	–	–	–	–	16	1.0	0.5	> 25.0

EC: *E. coli* (ATCC 35218); KP: *Klebsiella pneumoniae* (ATCC 700603), PA: *P. aeruginosa* (ATCC 27853); PV: *Proteus vulgaris* (ATCC 6896); [a]: Values were given as mean of the triplicate including the diameter of well (9 mm); *: Streptomycin used as positive control of this assay; –: No activity.

Table 3
MIC and minimum fungicidal concentration of the crude extracts and isoflavonoid compounds against the fungal ATCC strains.

Fungal strains	Activity (μg/mL)	Extracts of *M. ferruginea*			Isoflavonoid compounds			Standard antibiotics	
		Chloroform	Methanolic	Water	Barbigerone	Calopogonium isoflavone-A	Durmillone	Amphotericin B	Nystatin
C. albicans (ATCC 10231)	MIC	256	256	256	256	256	> 1 024	< 0.5	2
	MFC	512	256	> 1 024	256	512	> 1 024	< 0.5	2
C. neoformans (ATCC 66031)	MIC	128	512	256	256	256	> 1 024	< 0.5	1
	MFC	256	> 1 024	512	256	256	> 1 024	< 0.5	1

MFC: Minimum fungicidal concentration.

Table 5
Antileishmanial activity of seed extracts and isolated isoflavones against *L. donovani* promastigotes.

Test samples		IC_{50} values[$]
Extracts[#] (μg/mL)	Water	> 500
	Methanolic	37.6 ± 0.5
	Chloroform	182.8 ± 0.2
Compounds (μmol/L)	Barbigerone	12.4 ± 0.5
	Calopogonium isoflavone-A	87.3 ± 0.1
	Durmillone	8.2 ± 0.7
	Amphotericin B[$]	0.4 ± 0.2

[#]: Extracts of *M. ferruginea*; [§]: IC_{50} values (measured in μg/mL for extracts and μmol/L for compounds) were represented as mean ± SE of three independent determinations; [$]: Amphotericin B was used as a positive control of this assay.

The isolated isoflavonoids were also tested for antileishmanial activity (Table 5). Durmillone and barbigerone were found to be potentially active against the promastigotes of *L. donovani* at IC_{50} values of 8.2 and 12.4 μmol/L respectively. Calopogonium isoflavone-A was comparatively less active (IC_{50} 87.3 μmol/L).

3.6. DNA-fragmentation effect of isoflavonoids

Comet assay is a sensitive method for DNA strand break detection in individual cells, and commonly used to identify certain chemical agents which bind to DNA and caused spontaneous disintegration under the physiological conditions[22]. In present study, comet assay was employed to evaluate the DNA damaging activity of the isoflavonoids on PBMCs. Figure 2 (C to H) represented the fluorescence microscopic pictures of comet assay, performed on the PBMC, treated at two different doses (1 μmol/L and 1 mmol/L) of barbigerone, calopogonium isoflavone-A and durmillone. The untreated (Figure 2A), and hydrogen peroxide treated (Figure 2B) cells served as the negative and positive controls respectively. Each image was analysed by Tritek comet software to quantify the (i) percentage of DNA present in comet tail; (ii) percentage of DNA present in comet head; (iii) comet tail length in μm, and (iv) tail moment (= tail length × percentage of DNA in tail), as given in Figure 3 and Table 6. Here, tail moment incorporated the relative measurements of migrating DNA in terms of length of the comet tail (reflected by the smallest detectable size) and the number of DNA fragments (represented by the staining intensity of DNA in the tail). Statistical comparison of each set of data with respect to the negative control was done by Student's *t*-test.

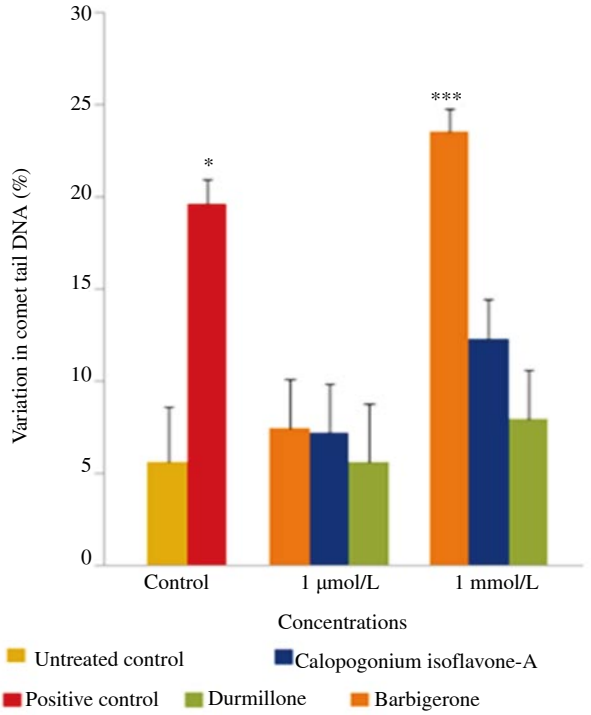

Figure 3. Estimation of the percentage of DNA present in comet tail. About 100 μmol/L hydrogen peroxide treated PBMCs were taken as positive control. Data were presented as mean ± SE. [*]: $P < 0.05$ and [***]: $P < 0.001$ were considered as significant difference compared to the untreated control group.

The treatment with H_2O_2 (100 μmol/L) showed significant change in the comet scores, indicating extensive damage of nucleic acid content in the cells. Thus, a decrease was observed in the percentage in comet head DNA [(80.30 ± 0.30)] with increase in the comet tail length [(11.70 ± 0.50) μm] in Table 6. More or less similar data, (76.40 ± 0.30) and (3.09 ± 0.70) representing head DNA (%) and tail length (μm) respectively were obtained when the cells were treated with 1 mmol/L of barbigerone, although this compound did not show significant damage at the lower concentration of 1 μmol/L (Figure 1C). However, calopogonium isoflavone-A and durmillone did not show DNA damage at the tested concentrations of 1 μmol/L and 1 mmol/L as no significant difference in tail moment was observed (Figure 2 E to H). Figure 3 depicted the graphical representation of percentage of DNA content in the comet tail.

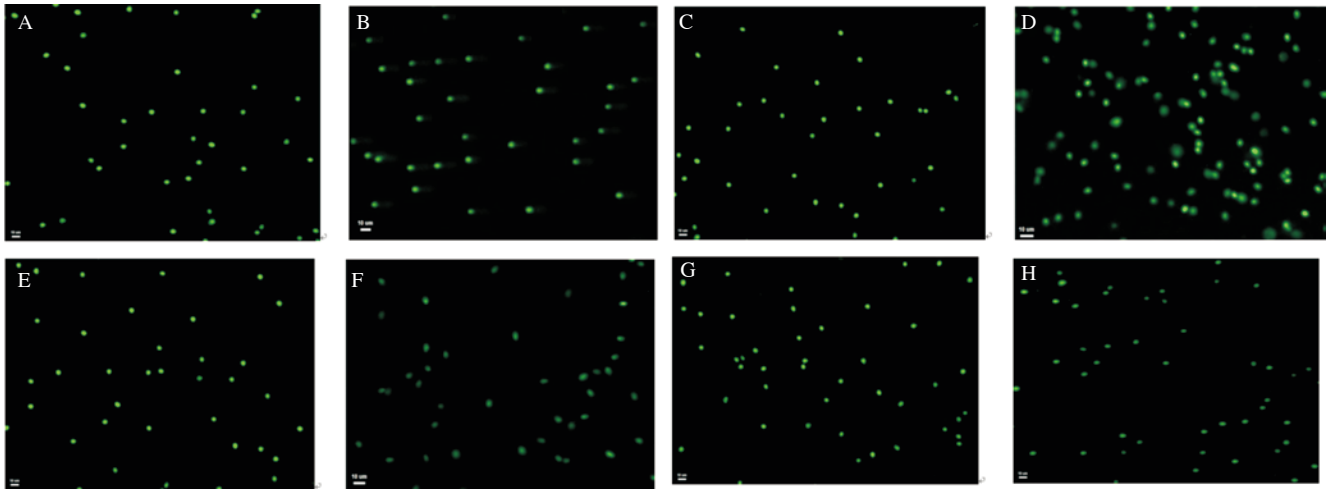

Figure 2. Comet assay for estimation of DNA damage in PBMCs; A: Untreated; B: Treated with 100 μmol/L hydrogen peroxide ; C: Treated with 1 μmol/L barbigerone; D: Treated with 1 mmol/L barbigerone; E: Treated with 1 μmol/L calopogonium isoflavone-A; F: Treated with 1 mmol/L calopogonium isoflavone-A; G: Treated with 1 μmol/L durmillone; H: Treated with 1 mmol/L durmillone cells.

Table 6
Evaluation of DNA damaging effect of the tested isoflavonoids on PBMC by comet assay.

Tested items	Barbigerone		Calopogonium isoflavone A		Durmillone		Positive control	Negative control
	1 μmol/L	1 mmol/L	1 μmol/L	1 mmol/L	1 μmol/L	1 mmol/L	100 μmol/L H_2O_2	–
% DNA in head	92.50 ± 0.20	76.40 ± 0.30***	92.70 ± 0.20	87.60 ± 0.30	94.30 ± 0.20	92.00 ± 0.20	80.30 ± 0.30*	94.30 ± 0.20
Tail length (μm)	0.80 ± 1.40	3.09 ± 0.70***	0.96 ± 1.90	1.50 ± 1.50	0.60 ± 2.20	1.00 ± 1.90	11.70 ± 0.50***	0.60 ± 1.90
Tail moment	0.60 ± 3.00	2.89 ± 1.50**	1.10 ± 2.90	1.70 ± 2.40	0.70 ± 4.00	1.20 ± 3.70	7.00 ± 1.80**	0.60 ± 4.10

Values were represented as mean ± SE. In test samples, positive control was compared with negative control data by independent, two tailed, Student's *t*-test. *: $P < 0.05$, which was statistically significant; ** and ***: indicated respective P values of < 0.01 and < 0.001, which was considered as statistically highly significant.

4. Discussion

More than 200 species of *Millettia* are distributed over the world, mostly in tropical Africa and some temperate regions in South America and Asia. Ethnobotanical surveys were conducted to note the multifarious uses of the endemic species in Ethiopian culture[1,11]. Traditionally, the indigenous people use aerial parts of *Millettia* plants as fishing poison and protecting stored food-grains from insects, which appear to be compatible with the typical presence of rotenoid compounds found in *M. ferruginea* and other species[3]. Most of the published literature on *Millettia* involved phytochemical analysis of the plant samples and this genus was found to contain a rich variety of structurally distinct isoflavonoids[5,15,23,24]. Unfortunately, these compounds have not been studied adequately regarding their prospective pharmacological properties. However, recently, isoflavonoids from *Millettia* have been reported for antiestrogenic, antiinflammatory and analgesic activities which have protective effects on myocardial ischemia[12,15,25,26]. Based on the traditional knowledge, the possibility of finding antiprotozoal compounds from *Millettia* sp. of plants in Africa was also indicated by Yenesew *et al*.[16]. More recently, Rajemiarimiraho *et al*. carried out extensive fractionation of *Millettia richardiana* bark and found potential antifungal and antiprotozoal activity in the crude extracts against *Candida* spp., *Plasmodium falciparum*, *Trypanosoma brucei brucei* and *L. donovani*[17]. However, no active compound was designated for the inhibitory activity observed in the solvent fractions prepared with aqueous and organic solvents.

Most published literatures on *Millettia* involved phytochemical analysis and were reported that *Millettia* spp. contains various phenolic compounds belonging to the groups of flavonoids, alkaloids, rotenoids, diterpenes and pterocarpans[5,15,23,24]. Initially, the isoflavonoid durmillone was isolated from the seeds of *Millettia dura* and *M. ferruginea*[4,27]. Members of the genus *Millettia* including *M. ferruginea* also contain substantial amounts of other isoflavones, like barbigerone and calopogonium isoflavone-A[5,12,16]. In our study, isolation and purification of the three isoflavonoids, namely, barbigerone, calopogonium isoflavone-A and durmillone were carried out to evaluate their efficacy against reference strains of bacteria (Table 2), fungi (Table 3), tubercle bacilli (Table 4) and leishmanial parasite (Table 5).

To start with, antibacterial assessment of *M. ferruginea* seeds was performed, and it was found that chloroform extract of *M. ferruginea* and methanol extract of *M. ferruginea* could inhibit the growth of Gram-negative strains more effectively than the Gram-positive bacteria, whereas no activity was observed in water extract of *M. ferruginea* (Table 1). This observation was encouraging as Gram-negative bacteria by virtue of their protective cell wall consisting of a thick lipopolysaccharide layer and are known to be less susceptible to therapeutics. In fact, Gram-negative bacteria possess the genetic ability to transmit and acquire resistance to multiple antibiotics and created serious public health concerns worldwide[28]. Therefore, we decided to test the water-insoluble isoflavonoid constituents of *M. ferruginea*, *viz.*, barbigerone, calopogonium isoflavone-A and durmillone against Gram-negative ATCC strains. The results in Table 2 showed that the compounds (MIC of 0.5 μmol/L) contributed to the antibacterial activity of water and methanol extracts of *M. ferruginea*. In addition, methanol and water extracts of *M. ferruginea* were also found to possess antiparasitic property with IC_{50} values of 37.6 and 182.8 μg/mL respectively and exhibited against *L. donovani* promastigotes, while water of *M. ferruginea* was found to be inactive (Table 5). The constituent isoflavonoid compounds were tested similarly, when durmillone (IC_{50} = 8.2 μmol/L) and barbigerone (IC_{50} = 12.4 μmol/L) showed marked antileishmanial activity and calopogonium isoflavone-A was less active (IC_{50} = 87.3 μmol/L) in comparison. However, none of the tested samples were found to be significantly active against the two tested yeasts and *M. tuberculosis* H_{37}Ra strain (Tables 3 and 4).

However, there was an obvious need to evaluate toxicity of these compounds against human primary cells before their consideration as clinical agents. For this purpose, comet assay was employed to study the DNA damaging activity of the isoflavonoids on PBMC. This technique is useful for testing genotoxicity of chemicals and pharmaceuticals[29]. Comet parameters (percent head DNA, percent tail DNA, tail length and tail moment) indicated a dose- dependent increase in DNA migration in PBMC (Figure 3 and Table 6), which indicates the extent of DNA damage due to the exposure of compounds as compared to control cells (Figure 2A). A comet like tail suggests the presence of a damaged DNA strand that migrates when electrophoreses was done. The tail length and percentage of DNA present in tail increase with the extent of DNA damage. The results in Figures 2 and 3, and Table 6 indicated that among the three tested compounds, only barbigerone exhibited maximum DNA damage in PBMC at a higher dose of 1 mmol/L (Figure 3 and Table 6). However, the percentage of tail DNA remained unaltered as compared with untreated control, when cells were treated with the lower concentration (1 μmol/L) of isoflavonoids (Figure 3). Thus, it could be concluded that inhibitory dose range of the compounds did not induce DNA damage in human PBMC. This observation was noteworthy as all compounds exhibited antibacterial (MIC < 1 μmol/L; Table 2) and antileishmanial (IC_{50} < 100 μmol/L; Table 5) activity at comparatively lower concentration to exert significant toxic effect towards the host cells (Table 6). Hence they could be considered for prospective antimicrobial application.

In conclusion, antibacterial efficacy of *M. ferruginea* seed extracts (water and methanol) was observed against both Gram-positive and

Gram-negative ATCC strains. Moreover, barbigerone, calopogonium isoflavone-A and durmillone isolated from the seeds were found to exhibit inhibitory activity against Gram-negative ATCC strains and *L. donovani* promastigotes for the first time. Further, comet assay was conducted on PBMC and pretreated with the three isoflavonoids in order to evaluate the possibility of DNA fragmentation induced by the compounds. The result showed that the compounds were safe to be considered for further therapeutic application.

Conflict of interest statement

We declare that we have no conflict of interest.

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References

[1] Andualem B, Gessesse A. Proximate composition, mineral content and antinutritional factors of Brebra (*Millettia ferruginea*) seed flour as well as physicochemical characterization of its seed oil. *Springerplus* 2014; **3**: 298.

[2] Andualem B, Gessesse A. Production and characterization of biodiesel from Brebra (*M. ferruginea*) seed non-edible oil. *Biotechnology* 2012; **11**(4): 217-24.

[3] Clark EP. The occurrence of rotenone and related substances in the seeds of the Berebera tree. A procedure for the separation of deguelin and tephrosin. *J Am Chem Soc* 1943; **65**: 27.

[4] Highet RJ, Highet PF. The structure of two isoflavones from Abyssinian Berebera tree. *J Org Chem* 1967; **32**(4): 1055-8.

[5] Dagne E, Bekele A. *C*-prenylated isoflavones from *Millettia ferruginea*. *Phytochemistry* 1990; **29**(8): 2679-82.

[6] Jembere B, Namukobe J, Kiremire BT, Dagne E. Extractives of *Millettia ferruginea*, *Tephrosia vogellii* and *Tephrosia pentaphylla* against the bean bruchid, *Zabrotes subfaciatus* Ethiopain head cabbage field. *SINT: Ethiop J Sci* 2007; **30**: 49-54.

[7] Mulatu B. Contact bioassay of an endemic plant to Ethiopia on three aphid species. *Ethiop J Biol Sci* 2007; **6**(1): 51-62.

[8] Perumalsamy H, Jang MJ, Kim JR, Kadarkarai M, Ahn YJ. Larvicidal activity and possible mode of action of four flavonoids and two fatty acids identified in *Millettia pinnata* seed toward three mosquito species. *Parasit Vectors* 2015; **8**: 237.

[9] Tilahun B, Azerefegne F. Efficacy of the aqueous crude extract of *Millettia ferruginea* (Fabaceae) on the maize stemborer *Busseola fusca* (Lepidoptera: Noctuidae) in the field. *Int J Trop Insect Sci* 2013; **33**(4): 256-63.

[10] Choudhury MK, Shiferaw Y, Hussen A. Toxicity of *Millettia ferruginea* darasana (family: Fabaceae) against the larvae and adult ticks of *Amblyomma variegatum* Fabricius a three-host tick in cattle. *J Parasit Dis* 2015; **39**(2): 298-302.

[11] Mesfin F, Demissew S, Teklehaymanot T. An ethnobotanical study of medicinal plants of Wonago, Woreda, SNNPR, Ethiopia. *J Ethnobiol Ethnomed* 2009; **5**: 28.

[12] Ito C, Itoigawa M, Kumagaya M, Okamoto Y, Ueda K, Nishihara T, et al. Isoflavonoids with antiestrogenic activity from *Millettia pachycarpa*. *J Nat Prod* 2006; **69**(1): 138-41.

[13] Yang JH, Hu J, Wan L, Chen LJ. Barbigerone inhibits tumor angiogenesis, growth and metastasis in melanoma. *Asian Pac J Cancer Prev* 2014; **15**(1): 167-74.

[14] Ndemangou B, Sielinou VT, Vardamides JC, Ali MS, Lateef M, Iqbal L, et al. Urease inhibitory isoflavonoids from different parts of *Calopogonium mucunoides* (Fabaceae). *J Enzyme Inhib Med Chem* 2013; **28**(6): 1156-61.

[15] Ye H, Wu W, Liu Z, Xie C, Tang M, Li S, et al. Bioactivity-guided isolation of anti-inflammation flavonoids from the stems of *Millettia dielsiana* Harms. *Fitoterapia* 2014; **95**: 154-9.

[16] Yenesew A, Derese S, Midiwo JO, Oketch-Rabah HA, Lisgarten J, Palmer R, et al. Anti-plasmodial activities and X-ray crystal structures of rotenoids from *Millettia usaramensis* subspecies *usaramensis*. *Phytochemistry* 2003; **64**(3): 773-9.

[17] Rajemiarimiraho M, Banzouzi JT, Nicolau-Travers ML, Ramos S, Cheikh-Ali Z, Bories C, et al. Antiprotozoal activities of *Millettia richardiana* (Fabaceae) from Madagascar. *Molecules* 2014; **19**(4): 4200-11.

[18] Debnath S, Dey D, Hazra S, Ghosh S, Ray R, Hazra B. Antibacterial and antifungal activity of *Terminalia arjuna* Wight & Arn. bark against multi-drug resistant clinical isolates. *J Coast Life Med* 2013; **1**(4): 315-21.

[19] Dey D, Ray R, Hazra B. Antitubercular and antibacterial activity of quinonoid natural products against multi-drug resistant clinical isolates. *Phytother Res* 2014; **28**(7): 1014-21.

[20] Dey D, Ray R, Hazra B. Antimicrobial activity of pomegranate fruit constituents against drug-resistant *Mycobacterium tuberculosis* and β-lactamase producing *Klebsiella pneumoniae*. *Pharm Biol* 2015; **53**(10): 1474-80.

[21] Hazra S, Ghosh S, Debnath S, Seville S, Prajapati VK, Wright CW, et al. Antileishmanial activity of cryptolepine analogues and apoptotic effects of 2,7-dibromocryptolepine against *Leishmania donovani* promastigotes. *Parasitol Res* 2012; **111**(1): 195-203.

[22] Singh NP. The comet assay: reflections on its development, evolution and applications. *Mutat Res Rev Mutat Res* 2016; **767**: 23-30.

[23] Yu WW, Jin C, Shuang PC, Liao H, Zhang L. [Isoflavones and flavans from *Millettia nitida* var. hirsutissima]. *Zhongguo Zhong Yao Za Zhi* 2015; **40**(12): 2363-6. Chinese.

[24] Deyou T, Gumula I, Pang F, Gruhonjic A, Mumo M, Holleran J, et al. Rotenoids, flavonoids, and chalcones from the root bark of *Millettia usaramensis*. *J Nat Prod* 2015; **78**(12): 2932-9.

[25] Huo X, Zhang L, Gao L, Guo Y, Zhang L, Li L, et al. Antiinflammatory and analgesic activities of ethanol extract and isolated compounds from *Millettia pulchra*. *Biol Pharm Bull* 2015; **38**(9): 1328-36.

[26] Huang J, Zhang X, Qin F, Li Y, Duan X, Jian J, et al. Protective effects of *Millettia pulchra* flavonoids on myocardial ischemia *in vitro* and *in vivo*. *Cell Physiol Biochem* 2015; **35**(2): 516-28.

[27] Ollis WD, Rhodes CA, Sutherland IO. The extractives of *Millettia dura* (Dunn): the constitutions of durlettone, durmillone, milldurone, millettone and millettosin. *Tetrahedron* 1967; **23**(12): 4741-60.

[28] Lushniak BD. Antibiotic resistance: a public health crisis. *Public Health Rep* 2014; **129**(4): 314-6.

[29] Araldi RP, de Melo TC, Mendes TB, de Sá Júnior PL, Nozima BH, Ito ET, et al. Using the comet and micronucleus assays for genotoxicity studies: a review. *Biomed Pharmacother* 2015; **72**: 74-82.