

Article

Extractability of Rutin in Herbal Tea Preparations of *Moringa stenopetala* Leaves

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Abstract: The study examined the comparative rutin contents and antioxidant potentials of the two closely related *Moringa* species: the Ethiopian (*Moringa stenopetala*) and Indian *Moringa* (*M. oleifera*). It is demonstrated that *M. stenopetala* leaves extract was a far superior (more than five-fold better) antioxidant than *M. oleifera*. Rutin was the principal constituent of *M. stenopetala* leaves while the compound was not detected in the leaves of *M. oleifera*. Quantitative HPLC-based analysis of *M. stenopetala* leaves revealed the rutin level at a respectable $2.34\% \pm 0.02\%$ (on dry weight basis), which is equivalent to many commercial natural sources of this highly sought-after bioactive compound. Comparative analysis of rutin in some common herbal tea preparations of *M. stenopetala* leaves revealed that it is readily extractible with the highest amount obtained ($98.8\% \pm 2.4\%$) when the leaves (1 g) were boiled in water (200 mL). For a large-scale exploitation of rutin, a fast and economically-viable isolation approach using solid phase extraction followed by crystallization or flash chromatography is outlined. Overall, the Ethiopian *Moringa* is distinctively different from the Indian *Moringa* and could be exploited as an industrial source of rutin for nutritional and/or medical uses.

Keywords: *Moringa stenopetala*; *Moringa oleifera*; Ethiopian *Moringa*; Indian *Moringa*; antioxidant; rutin; rutin extraction; industrial source of rutin

1. Introduction

The Ethiopian Moringa *Moringa stenopetala* is incredibly similar to the Indian Moringa *M. oleifera* in many respects. In both cases, they are called “The Miracle Tree” owing to their multipurpose applications including as a source of food, medicine, edible oils, biofuel and applications for water sanitations [1,2]. While the chemistry and pharmacology of the Indian Moringa along with its usage have been exhaustively studied, the scientific evidence to justify the multipurpose usage of *M. stenopetala* has only emerged in recent years. As with the Indian Moringa, *M. stenopetala* is now introduced in many tropical countries for its popular uses but its origins are the southern regions of Ethiopia and Northern Kenya. Commonly known as cabbage-tree in English or locally as *aleko* or *shiferaw*, *M. stenopetala* is widely used by many Ethiopian communities as a source of diet. In this regard, one of the greatest socioeconomic impacts of the plant lies in its ability to grow in poor soil and achieving harvest in a short amount of time. This quality of the plant has also been noticed in recent years by many international aid agencies as funding was made available for a large-scale Moringa farming in areas vulnerable to frequent droughts. The exemplary work by Global Team for Local Initiatives (GLTI) in collaboration with the Horn of Africa Regional Environment Center & Network (HoA-RECEN) and the UN’s Food & Agriculture Organization (FAO) is worth mentioning. GLTI so far has been assisting in the delivery of around 145,000 Moringa trees to the southern regions of Ethiopia such as the Hamar and Dasenech families in the South Omo Zone [3]. Interestingly, a number of nutritional composition studies on the leaves so far (e.g., [4,5]) have been in favour of the plant’s usage as a dietary source. The message of Moringa utilisation also appeared to be well received by consumers, and both fresh and dried powdered leaves of *M. stenopetala* are now widely sold in cities of Ethiopia and abroad as nutritional supplements. The plant is also extensively employed in the Ethiopian and Kenyan traditional medicine for treating a range of illnesses such as diabetes, hypertension, stomach pain, malaria, leishmaniasis, leprosy, epilepsy, diarrhoea, asthma, colds, as an anthelmintic, emetic and wound healing [6]. Some of these therapeutic claims for the various parts of the plant have been validated through pharmacological studies including antimicrobial [7–9], antihypertensive [10], antispasmodic [11] and blood glucose and other biochemical parameters assessments [12–14]. To date, the most extensive commercial usage of the Ethiopian Moringa as a nutritional supplement and medicine is through consumption of the dried leaves as a herbal tea.

A recent study by Habtemariam [15] did look into the active principles responsible for the Ethiopian Moringa’s potential usage as an antioxidant and treatment for diabetes and associated diseases. The study revealed that rutin is the principal constituent of the leaves along with a very small amount of neochlorogenic acid. Since then, the therapeutic potential of rutin for diabetes and associated diseases has been reviewed in detail [16] and there is no doubt that rutin’s abundance in *M. stenopetala* leaves plays a major role in the nutritional and medicinal value of the plant. Rutin, however, is poorly soluble in water and highly soluble in polar solvents like alcohols. Its presence in the methanol extract as described by Habtemariam [15] does not thus necessarily mean that it is extractable by water in herbal tea preparations. The present study was therefore designed to assess the extractability of rutin in herbal teas and also develop a rapid cost-effective isolation procedure for large-scale exploitation of rutin from the plant. Comparative antioxidant activity and HPLC profile analysis of the two closely related Moringa species and the implication of the data in adulteration of Moringa products are also discussed.

2. Experimental Section

2.1. Chemicals

DPPH, 5,5-dithiobis (2-nitrobenzoic acid) (DTNB) and rutin standard were purchased from Sigma (Sigma-Aldrich Chemical Company, Dorset, UK). HPLC solvents and other reagents were obtained from Fisher Scientific UK Ltd. (Loughborough, UK). All other chemicals and reagents were of analytical grade and solutions were freshly prepared before use.

2.2. Plant Material Samples

Powdered leaves of *M. stenopetala* were purchased from various sources of Ethiopian herbal suppliers. As described by Habtemariam [15], HPLC-based analysis has shown that these products were incredibly similar and leaves powder from only one source (Abadir Supermarket, Arat Kilo, Addis Ababa, Ethiopia) obtained in September 2013 were used. The leaves of healthy *M. oleifera* plants were collected in June 2013, from Kottayam district, Kerala state, India. The location of the plant population lies between 09°34'983" north latitude and 76°31'189" east longitude, 3 km east of Kottayam town. A voucher specimen numbered 3766 was deposited at NKP Vaidyar Ayurvedic Research Foundation Herbarium, NUPAL Road, Kadavanthra, Ernakulum, Kerala state.

2.3. Extraction

2.3.1. Extractability of Rutin from *M. stenopetala* Leaves

Accurately weighed dried leaves samples of *M. stenopetala* (1 g) were placed in reaction glasses and refluxed on temperature-controlled hot plates. The system was left for the indicated period for extraction with either water, methanol or methanol:water (50:50). In some experiments, magnetic stirrers were used to enhance extractability. After completion of the extraction period, samples were taken, filtered and analysed by HPLC. All experiments were repeated at least 4 times.

2.3.2. Determination of the Maximum Amount of Extractible Rutin

In order to determine the maximum level of rutin that can be extracted from the leaves of *M. stenopetala*, dried powder leaves (1 g) were extracted by methanol as described above. The process was repeated 5 times until no rutin peak was detected by HPLC. The total amount of rutin extracted by this procedure was taken as the maximum extractible rutin and was expressed as 100% extractability.

2.3.3. Large Scale Extraction for Activity Studies

The powdered leaves (50 g) samples were extracted twice by soaking in 1 L methanol for two weeks. After removing the extract and filtration, samples were dried under reduced pressure using rotary evaporator to yield crude extracts.

2.4. HPLC Analysis

The HPLC analysis was based on an Agilent 1200 series gradient system composed of degasser (G1322A), quaternary pump (G1322A), auto sampler (G1329A), thermostat column compartment (G1316A) maintained at 25 °C and a diode array detector (G1315D). Different plant sample preparations or dilutions of the standard (rutin) were injected (20 µL) onto a reverse phase column (Agilent—Eclipse XDB-C18, 5 µm, 4.6 × 150 mm). The mobile phase was a mixture of water (A) and methanol (B). The composition of the mobile phase at a flow rate of 1 mL/min was rising from 10% to 90% B over a period of 50 min.

2.5. Isolation of Rutin

2.5.1. Solid Phase Extraction (SPE)

Solid phase extraction was carried out by using CHROMABOND C18 EC octadecyl silica polypropylene columns (endcapped adsorbent weight of 100 mg in 1 mL volume; Macherey-Nagel brand) under a vacuum set up. The columns were first conditioned with 10 ml of methanol followed by 10 mL of the elution buffer which was water containing 0.1% acetic acid. Rutin samples (5 mg/mL) were then loaded onto the columns and washed with the elution solvent (10 mL). The adsorbed rutin was recovered by eluting columns with methanol (10 mL). The level of purity of rutin was analysed by HPLC.

2.5.2. Combiflash Chromatography System

RediSep C18 gold column (100 g, Presearch, Hampshire, UK) attached to a Teledyne Isco flash chromatography system was used to isolate rutin from *M. stenopetala*. The solvent system was composed of methanol and water: A linear gradient started from 10% methanol to 40% over a period of 15 min and then raising methanol to 90% at 20 min. The flow rate was maintained at 60 mL/min and the chromatogram was monitored by using dual wavelength of 214 and 254 nm. The fractions of *M. stenopetala* obtained from the SPE process were subjected to this chromatographic system to isolate highly purified rutin in just one-step work up.

2.5.3. Crystallization of Rutin

Fractions of rutin samples were dried using rotary evaporator under reduced pressure and low temperature. Small volume of water was added to the dried sample under heat. After filtration, samples were kept at 4 °C until crystals develop.

2.6. DPPH Radical Scavenging

The antioxidant activity of test samples was measured by using our established microtitre-based DPPH assay [17]. Briefly, DPPH solution (0.1 mM, in methanol) was incubated with varying concentrations of test compounds for 20 min at room temperature and the blue colour absorbance of the resulting solution was read against a blank using Multiscan EX Reader (Thermo Labsystems, Altrincham, UK).

2.7. General Phytochemical Analysis Methodology

1D (^1H NMR, ^{13}C NMR) and 2D-NMR (COSY, NOESY, HMQC and HMBC) spectra were obtained on a JEOL 500 MHz instrument as described previously [15]. Accurate mass measurement over the full mass range of m/z 50–2000 was recorded using the high resolution mass spectroscopy instrument, Thermofisher LTQ Orbitrap XL (Thermofisher Scientific, UK), with an electrospray ionisation probe. Samples diluted into MeOH + 10% NH₄OAc were delivered by using NanoMate. The Nano-electrospray analyses were performed in positive ionisation mode at a temperature of 200 °C, sheath gas flow of 2 units and capillary (ionising) voltage at 1.4 kV. The accurate mass measurements obtained from this system were far better than 3 ppm.

2.8. Statistical Analysis

All data are presented as means and SEM values. Where appropriate, the significance of difference between two means was analysed by using unpaired *t*-test.

3. Results and Discussion

Rutin is a common dietary flavonoid widely distributed in the plant kingdom. It is also present in plant-derived beverages and foods as well as numerous medicinal and nutritional preparations. The story of rutin in plants goes as far back as 1842 when it was discovered from *Ruta graveolens*. The knowledge of its presence in various plants such as elder (*Sambucus canadensis* L.) also goes as far back as the beginning of the 20th century [18]. Today, rutin is among the most popular natural flavonoids known for its multifunctional nutritional and therapeutic uses [16,19]. As far as commercial exploitation of rutin is concerned, however, very few plants store it in large amounts to merit the cost of its extraction from natural sources. In this regard, the most popular commercial source of natural rutin to date is buckwheat with two species—*Fagopyrum esculentum* and *Fagopyrum tataricum*—shown to be most important. The aerial parts of these plants, particularly flowers and leaves, have been found to contain from about 2 to 10% of rutin in dry weight [20–22]. Variability of rutin content based on different cultivars [23] and plant parts [24] as well as environmental conditions such as exposure to UV light [25] have also been reported. Although the rutin content of buckwheat is astonishingly good, commercial exploitation from this source is not without problems. For example, the best plant part, the inflorescence that may contain up to 12% in dry weight [24], is not a commercially viable source for rutin extraction. Allergic reactions including hypersensitivity such as asthma and gastrointestinal disorders have also been reported [26,27]. Furthermore, rutin in almost all cases is present in plants together with an array of related flavonoids. Its isolation and/or fortification for nutritional uses have therefore need to be balanced by the cost of fractionation/separation processes.

3.1. HPLC Profile of *M. stenopetala* and *M. oleifera*

As shown in Figure 1, the HPLC-based composition analysis of the methanol extract of *M. stenopetala* as reported by Habtemariam [15] is quite different from that of *M. oleifera*. While rutin was not detected at all in the tested *M. oleifera* sample, it is uniquely predominant in *M. stenopetala*. The lack of peaks other than rutin in the flavonoids region of *M. stenopetala* is particularly astonishing.

This finding is in agreement with previous reports where a number of flavonoids and their glucosides other than rutin predominates in the leaves extract of *M. oleifera*. For example, the study by Bennett *et al.* [28] has identified the major phenolic components other than glucosinolates in *M. oleifera* leaves as quercetin-3-*O*-glucoside and quercetin-3-*O*-(6''-malonyl-glucoside) and minor components as kaempferol-3-*O*-glucoside, kaempferol-3-*O*-(6''-malonyl-glucoside), chlorogenic acid and neochlorogenic acid. The overall major component of *M. oleifera* however remains to be glucosinolates that come within the first 5 min of the HPLC analysis time (Figure 1). As described previously [15], the concentration of rutin in *M. stenopetala* plant samples was analysed by using purified rutin as an external standard. For this analysis, plant samples were first extracted five times with methanol to make sure that all extractible rutin was accounted for. From three separate experiments, the mean level of rutin extractible from 1 g dry weight leaves was 23.39 ± 0.17 mg. The percentage of rutin content on a dry weight basis was thus $2.34\% \pm 0.02\%$. This data suggests that the Ethiopian Moringa, unlike *M. oleifera*, can be exploited as an alternative commercial source of rutin. Apart from buckwheat, which on a commercial-scale cultivars can yield about 5%, very few plants have been identified so far with rutin content of this magnitude. Industrial scale production of rutin from other sources includes *Eucalyptus macrorhyncha* and tobacco leaf but the high cost of production and low rutin content (less than 1.5%) have made them economically less attractive sources. Industrial production of rutin from *Ruta graveolens*, *Sophora japonica*, *Maranta leuconeura*, *Orchidantha maxillarioides*, *Strelitzia reginae*, *Canna indica*, *Canna edulis* and *Labisia pumila* among others has also been employed in recent years. With further research on careful selection of *M. stenopetala* varieties grown under different environmental conditions, it is likely that *M. stenopetala* could serve as a reliable alternative source of industrial-scale rutin production for nutraceutical and medical uses.

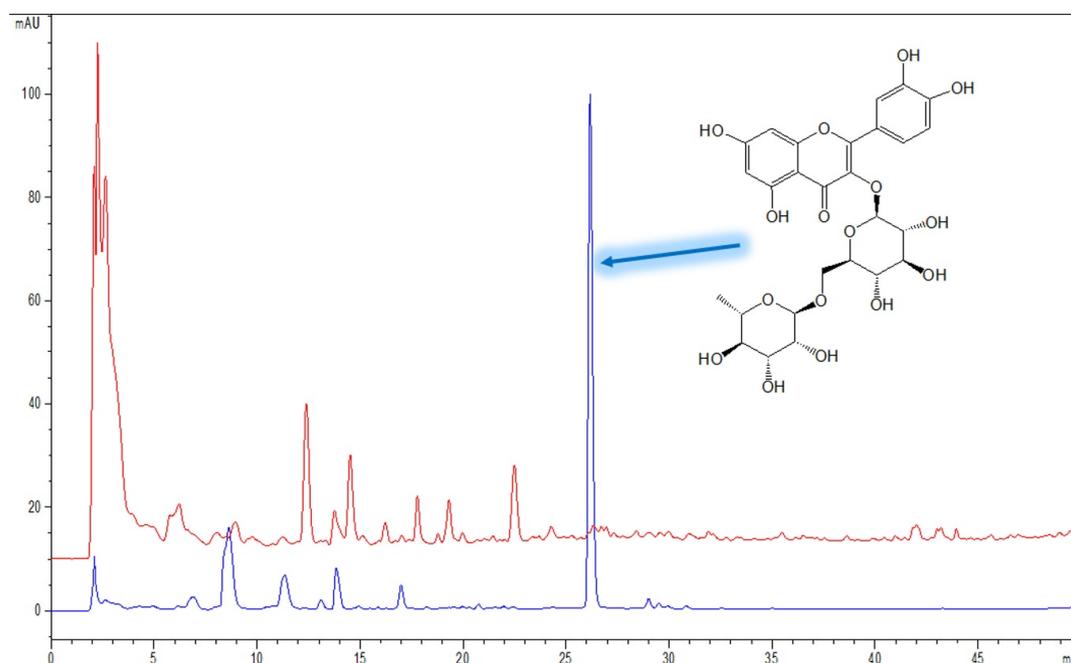


Figure 1. HPLC profiles of crude methanolic extracts of *M. oleifera* (top panel) and *M. stenopetala* (lower panel). The HPLC analysis was based on 5 mg/mL solution of crude leaves extracts as described in the experimental section. Notice the presence of rutin (structure shown) only in *M. stenopetala* extract.

3.2. Comparative Antioxidant Analysis of *M. stenopetala* and *M. oleifera*

The antioxidant activity of *M. oleifera* has been reported in numerous literature and that of *M. stenopetala* has been reported very recently by Habtemariam [15]. In line with the rutin-based distinction between the two Moringa species, the present study showed that potent antioxidant activity was evident only in *M. stenopetala* (Figure 2). Readers must bear in mind that *M. oleifera* is one of the most acclaimed natural antioxidant agents. If one has to closely examine the concentrations used in most of the reported literature, however, they would see they are very high, often in mM range as with nutritional standards [29,30]. In agreement with these findings, the present study (Figure 2) revealed that the full concentration-response curve for *M. oleifera* lies from sub-mM to mM range. The IC₅₀ values data in Table 1 also revealed that the Ethiopian Moringa is over five times more potent than the Indian Moringa. Although one has to be conscious about the possibility of better varieties or collection time of *M. oleifera* that may yield higher antioxidant effects, it is clear that *M. stenopetala* is far superior as an antioxidant agent. Since these two plants are now growing side by side in many countries and the common knowledge by people is that they are similar in their activities, an educational drive in product distinction both by traders and consumers is necessary.

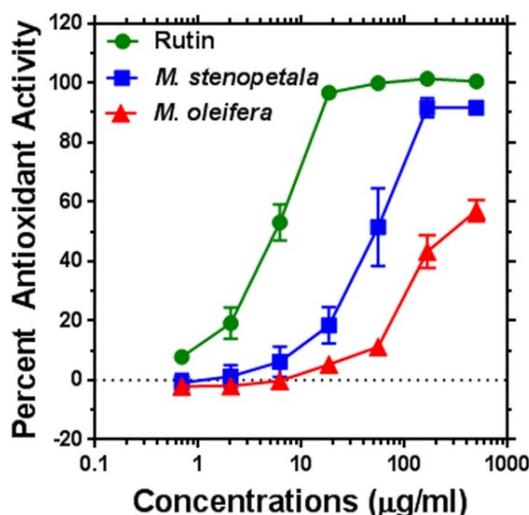


Figure 2. Comparative radical scavenging effect of *M. stenopetala*, *M. oleifera* and rutin. The DPPPH radical scavenging effect was assessed as described in the materials and methods section.

Table 1. Antioxidant effects of crude leaves extracts of *M. stenopetala* and *M. oleifera*. IC₅₀ values for the extract along with the standard rutin are shown. Data are means and SEM values from four separate experiments.

Treatment	IC ₅₀ Values (µg/mL)
<i>M. stenopetala</i> extract (methanol)	36.00 ± 6.47 *
<i>M. stenopetala</i> extract (water)	40.00 ± 6.12 *
<i>M. oleifera</i> extract (water)	215.0 ± 40.52
Rutin	5.00 ± 0.98

Data marked by (*) indicate not significantly different ($p > 0.5$) from each other.

3.3. Extractability and Quantification of Rutin in Herbal Teas

Rutin is a polar compound with high solubility in polar organic solvents like acetone and alcohols. Its solubility in water is, however, very disappointing with only about 0.13 g/L recorded in many studies [31,32]. Although extractability of solutes from crude mixtures is related to their solubility in a chosen solvent, the relationship between the two processes is not straightforward. As shown in Figure 3, the extractability of rutin by water and methanol was comparable (no statistical difference, $p > 0.05$). Hence, it is fair to conclude that rutin is extractable in the herbal tea preparation of *M. stenopetala*. The data also suggest that a cheap method of large-scale extraction of rutin from the plant using water can be employed.

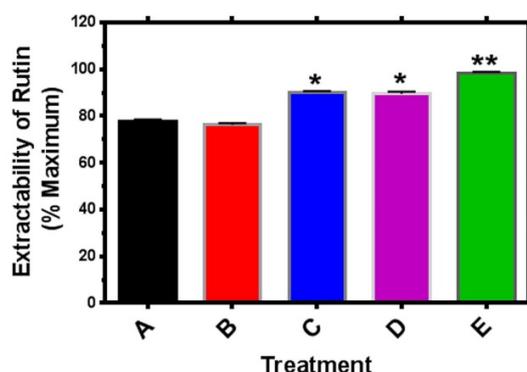


Figure 3. Extractability of rutin from the dried leaves of *M. stenopetala*. After the dried leaves were subjected to different extraction treatments, the amount of rutin extracted was quantified using HPLC from rutin standard curve. Values were converted into percentage by using the maximum extractable amount as 100%. A = Cold methanol maceration for 20 min; B = Cold water treatment for 20 min; C = Cold water treatment with stirring for 20 min; D = Boiling water (100 °C) added and left for 20 min; E = Boiling in water for 30 min at 250 °C setting. Data are mean and SEM values from four separate experiments.

In some herbal tea preparations, water is boiled and then added to the herb in a coffee cup. We imitated this by boiling water and increasing the temperature to 100 °C, and using it to soak the plant material in a flask for exactly 20 min. As one expects to see the extractability of rutin increase with temperature, the rutin content under this treatment was significantly higher than the cold treatment by either water or methanol (Figure 3). Interestingly, simple stirring of samples during the 20 min extraction period could also enhance the yield of rutin by the same magnitude as using boiling water (Figure 3). Since we also noticed some people choose to boil *Moringa* leaves in a pot to prepare their herbal teas, we imitated this scenario by boiling *M. stenopetala* leaves for 30 min. Under this latter treatment, the extractability of rutin was significantly higher than all treatment groups (Figure 3). Even though rutin is known to be unstable during hot water extraction, we did not notice any degradation during the 30 min treatment period (Figure 4). The maximum benefit of herbal tea preparation using *M. stenopetala* leaves is thus through the use of hot water as exemplified in our experiment. It is worth noting that there has been some studies that looked into the levels of carotenoids and ascorbic acid (Vitamin C) in fresh leaves of *M. stenopetala*. While carotenoids are generally resistant to cooking and only a moderate drop of 11% was noted, the loss of ascorbic acid by cooking could be drastic with over 90% loss [4]. Further

investigation is thus required to maintain the balance between rutin extraction in hot water preparations and potential loss of ascorbic acid and/or aroma from dried leaves of *M. stenopetala*.

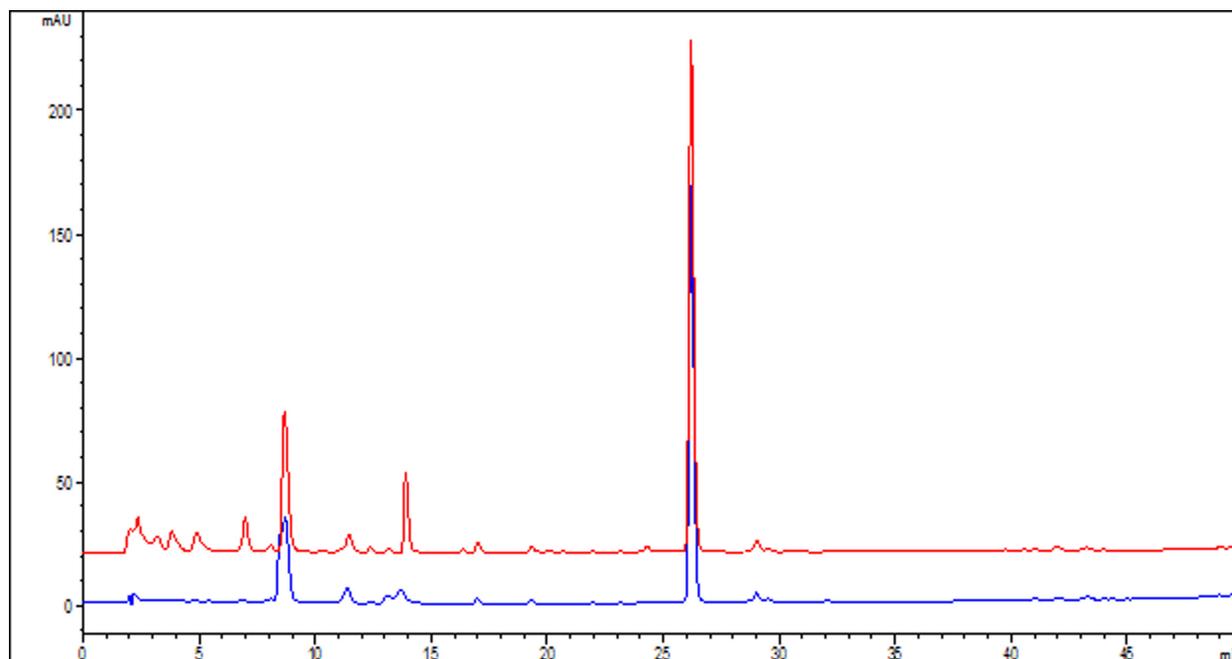
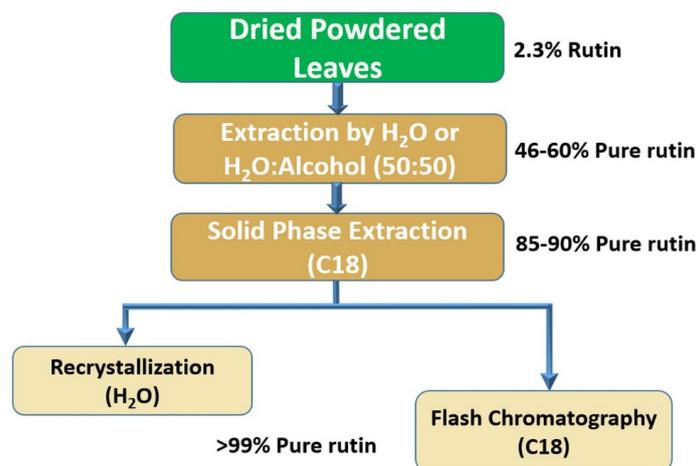


Figure 4. Comparative HPLC chromatograms of *M. stenopetala* leaves extracted by cold methanol and boiling in water. The top panel shows the leaves powder (1 g) boiled for 30 min and lower panel cold methanol for 20 min. Notice that no extra peaks were detected in the boiling preparation before or after the rutin peak (just after 26 min).

3.4. Isolation of Rutin

An efficient combiflash system of chromatography for the isolation of rutin from the methanol extract of leaves has previously been described by Habtemariam [15]. The employed method was based on initial fractionation of the crude extract followed by a one-step separation using a reverse-phase (C18) fast flash chromatography system. In the present study, an attempt was made to reduce the fractionation step and the cost of solvent-based extraction. As discussed above, water-based extraction especially in combination with heating can provide a better yield of rutin. Qualitatively, we also observed that the methanol, water and methanol:water (50:50) extracts have the same chromatographic profile with rutin purity (by HPLC, 280 nm) ranging from 46% to 60%. Crystallization of rutin from high-yielding extracts is always a possibility and we attempted this by dissolving dried crude extracts in the smallest possible volume of water under heat followed by refrigeration. The volume of crystal recovery under this condition over a weak period was, however, not sufficient enough to deem the methods as a viable procedure. A solution of rutin in water kept for weeks also shows some degree of degradation. We therefore opted to try another system of separation using SPE which is considered very cost-effective. Intestinally, SPE yielded rutin close to 90% purity in less than 30 min work-up (Scheme 1). Further purification for a highly purified rutin can be achieved by either a crystallisation process or a combiflash system [15]. The identification of the isolated compound as rutin was established through comprehensive spectroscopic methods including NMR and MS as

described previously [15]. The overall proposed approach for industrial-scale rutin purification from *M. stenopetala* leaves is shown in Scheme 1.



Scheme 1. Approach for rapid industrial-scale isolation of rutin from *M. stenopetala* leaves.

The antioxidant potential of the water extract of *M. stenopetala* leaves was assessed by using the DPPH radical scavenging assay in comparison with the rutin standard. As shown in Table 1, the water extract showed antioxidant activity comparable with the methanol extract ($p > 0.05$). The validation of antioxidant potential of herbal medicines in our laboratories is based on several in-house *in vitro* cell-free and cell-based assays [33–44] as well as *in vivo* models [45–54]. Since the principal active constituent of the methanol and water extracts of *M. stenopetala* leaves is the well-characterised antioxidant and bioactive agent rutin, further biological bioassays on the extracts were not necessary.

4. Conclusions

The present study clearly shows that the methanol and water extracts of *M. stenopetala* are distinctly different from the Indian Moringa *M. oleifera*. Rutin as the principal antioxidant component of the leaves of *M. stenopetala* is readily extractable in herbal tea preparations. The most productive way of extracting rutin in herbal teas is by boiling plant samples in water. The fact that rutin was found in the Ethiopian Moringa leaves at the level of $2.34\% \pm 0.02\%$ (on dry weight basis) implies its potential usage as a reliable alternative commercial source of this multifunctional bioactive natural compound.

Acknowledgments

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Author Contributions

S.H. conceived, designed, carried out the experiments and wrote the paper; G.V. has worked on the collection and identification of *M. oleifera*.

Conflicts of Interest

No competing financial interests exist.

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