



IN VITRO ANTIOXIDANT EFFECTS OF DICHLOROMETHANE – METHANOLIC BLEND EXTRACTS OF *Clutia abyssinica* AND *Maytenus obscura*

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ABSTRACT

The present study was designed to evaluate the *in vitro* antioxidant potential of Dichloromethane:-Methanolic blend leaf extracts of *Clutia abyssinica* and *Maytenus obscura*. This was determined using 1,1 –Diphenyl-2-Picrylhydrazyl (DPPH) scavenging assay, ferric reducing power, hydrogen peroxide scavenging assay and total phenolic content at various concentrations. Ascorbic acid was used as the standard. In addition, qualitative phytochemical screening of the extracts was done. The DCM-MeOH blend leaf extracts of both plants demonstrated a dose dependent increase in DPPH scavenging activities and total ferric reducing power. However, they demonstrated a dose dependent reduction in hydrogen peroxide scavenging activity. There were significant differences between the extracts and the standard in terms of antioxidant activities. Total phenol content correlated positively with the antioxidant capacities of the plant extracts. Phytochemical analysis showed presence of different secondary metabolites associated with antioxidant activities. In conclusion the DCM: MeOH blend extracts of *C. abyssinica* and *M. obscura* demonstrated remarkable *in vitro* antioxidant activities. Therefore, *Clutia abyssinica* and *Maytenus obscura* maybe be used as alternative or complementary sources of antioxidants for management of different health conditions that arise due to oxidative stress. The present study scientifically validates and supports the traditional use of these plants in the management of oxidative stress.

Keywords: oxidative stress, DPPH scavenging, ferric reducing power, total phenolic content, antioxidant activity.

INTRODUCTION

Living organisms that carry out aerobic metabolism are exposed to dangers associated with free radicals, reactive oxygen species (ROS) and reactive nitrogen species (RNS). Free radicals are unstable and reactive molecules released as by-products of aerobic metabolism or from exogenous sources such smoking and radiation [1]. Examples include super oxide radical, nitric oxide, hydrogen peroxide and hydroxyl radical [1]. At

moderate levels free radicals play different physiological functions in the body, such as in signal transduction and cell immunity [2, 3]. However, accumulation of free radicals leads to oxidation of different biomolecules in the body such as DNA, proteins and lipids, causing injuries to different body tissues thus, inducing oxidative stress [4]. All aerobic organisms have defense mechanisms to protect the body from harmful effects caused by the free radicals, failure of which results in oxidative damage [5].

Oxidative stress is a state of imbalance between oxidants and antioxidants. It is the main cause of several disease conditions such as diabetes, different types of cancers, cardiovascular diseases, inflammation and aging [6]. Thus, the need for antioxidants to manage oxidative stress. Antioxidants are compounds with the ability to prevent oxidation of other molecules in the body by free radicals [7, 8]. They react with free radicals and make

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them stable, hence, reducing their ability to react with different cell components [9].

Synthetic antioxidant supplements are used in the conventional management of oxidative stress. Such supplements include BHA-butylated hydroxyl anisole, BHT-butylated hydroxyl toluene and propyl gallate [10]. However, they are unaffordable, not easily accessible and are suspected to be responsible for liver damage and carcinogenesis in laboratory animals [11, 12]. There is, therefore, the need to develop more affordable, accessible and safe antioxidants. There has been increasing interest in search for antioxidants from natural sources because they are perceived to be safer and easily accessible [13]. Such plants include *Clutia abyssinica* and *Maytenus obscura*.

Maytenus obscura (A. Rich.) Cufod. commonly known as black seed oil or climbing staff tree, is a shrub that grows up to 10m with long slender branches that are reddish brown in colour [14]. It is native in India, but is also grows in Australia, China, Taiwan, Cambodia, Indonesia, Laos, Malaysia, Myanmar and Nepal. Traditionally it is used as an antiseptic, antiasthmatic, antitumor, antiulcer and in fertility-regulation [15]. On the other hand *Clutia abyssinica* Jaub. and Spach. is a shrub that belongs to the Euphorbiaceae family. It is commonly known as lightning bush or smooth fruited clutia. It is concisely of about 2m high with sparsely branched glabrous twigs [16]. It is native in Tanzania, Kenya, South Africa, Angola, Ethiopia and Zimbabwe [17]. Traditionally, its root extract is used to treat intestinal worms, influenza, colds and fever, and indigestion [17]. Moreover, the root extract is used in the management of habitual miscarriages [18]. *Clutia abyssinica* and *Maytenus obscura* have been used in management of different oxidative stress-related disease conditions. However their use has not been scientifically validated. It is against this background that this study was designed.

MATERIALS AND METHODS

Plant Collection

Fresh leaves of *Clutia abyssinica* were collected from their natural habitat in Kaptebee village, Turbo sub-county in Uasin Gishu County, Kenya, while fresh *Maytenus obscura* leaves were collected from Makunguru village, Mbeere North Sub-county, Embu County, Kenya. This was done with the help of traditional medicinal practitioners. The plant samples were sorted, cleaned and transported to Kenyatta University. They were taxonomically identified by an acknowledged taxonomist and voucher specimens were deposited in the Kenyatta University herbarium.

Sample Processing and Extraction

The leaves were dried under a shade until dry. They were then ground to powder using a mechanical grinder and stored at room temperature. The dried powder (400 g) of each plant was soaked for 24 hours in a 1L

mixture of dichloromethane and methanol in the ratio of 1:1. The mixture was then decanted into a clean dry conical flask and filtered using Whatman's filter paper No. 1 into another conical flask. The filtrate was then concentrated using a rotary evaporator at a temperature of 64°C to yield a solid residue.

Determination of DPPH Radical Scavenging Activity

The DPPH scavenging activity was done according to the protocol of [19] with some modifications. Different concentrations of the extracts (0.0625 to 1 mg/ml) were prepared in methanol. The same concentrations were also prepared for ascorbic acid, which was used as a standard. The extract (1 ml) was put in a test tube and 1 ml of methanol added followed by 0.5ml of 0.1 M DPPH. The mixture was shaken vigorously and left to stand for 5 minutes. A blank solution was prepared containing the same amount of methanol and DPPH. The absorbance of the resulting solution was measured at 517 nm using a spectrophotometer. The experiment was repeated three times. The DPPH radical scavenging activity was estimated based on the percentage of DPPH radical scavenged using the following equation:

$$\text{DPPH scavenging activity (\%)} = \frac{A_c - A_s}{A_c} \times 100$$

Where: A_s = absorbance of the sample

A_c = absorbance of the control

A curve was generated based on the percentage of DPPH radical scavenging activity using linear regression and it was used to calculate the half maximal inhibitory concentration (IC_{50}).

Determination of Total Ferric Reducing Power

The total reducing power of the extracts was determined as described in [20] with some modifications. Varying concentrations of the extracts (0.2 to 1 mg/ml in distilled water) were prepared, mixed with 2 ml phosphate buffer (0.2 M, pH 6.6) and 2 ml of 1% Potassium Ferricyanide [$K_3Fe(CN)_6$]. The mixture was then incubated at 50°C for 20 minutes, after which 2 ml of 10% Trichloroacetic acid (TCA) was added to the mixture. The mixture was then centrifuged for 10 minutes at 3000 rpm. The upper layer of solution (2 ml) was mixed with 2 ml of distilled water following which 0.5 ml of $FeCl_3$ (0.1%) was added and the reaction was allowed to stand for 10 minutes. The same procedure was done for ascorbic acid, which was used as a standard. The experiment was done in triplicates and absorbance was then measured at 700 nm using a spectrophotometer.

Determination of Hydrogen Peroxide Scavenging Activity

The hydrogen peroxide scavenging activities of the extracts was determined according to the procedure of [21]. The plant extracts were prepared in distilled water at different concentrations (0.00625 to 0.1 mg/ml), then

mixed with 1 ml of 40 mM H₂O₂ solution prepared in phosphate buffer (0.1 M, PH 7.4) and left to stand for 10 minutes at room temperature. This procedure was also done for ascorbic acid, which was used as a standard. The experiment was done in triplicates and the absorbance of the solution was taken at 560 nm against blank solution containing phosphate buffer without H₂O₂. The hydrogen peroxide scavenging activity of the extracts was calculated using the following equation:

Hydrogen peroxide scavenging activity (%)

$$= \frac{A_c - A_s}{A_c} \times 100$$

Where: A_s = absorbance of the sample

A_c = absorbance of the control

Determination of Total Phenolic Contents

The total phenolic contents of the extracts was done according to Folin-Ciocalteu method with some modifications. The extract (1 ml) was mixed with 2 ml of Folin-Ciocalteu reagent which was prepared by dilution with distilled water in a ratio of 1:10 v/v, after which 1 ml of 20% Sodium Carbonate (Na₂CO₃) was added. The mixture was shaken for 20 seconds and incubated at 40°C for 30 minutes. Absorbance was measured at 765 nm. Gallic acid was used for the generation of the standard curve. The total phenolic content was expressed as mg of gallic acid equivalents (GAE) per gram (g) of the dried sample.

Qualitative Phytochemical Screening

The plant extracts were subjected to qualitative phytochemical screening to find out the active compounds present, as described in [22, 23]. The secondary metabolites tested included alkaloids, tannins, flavonoids, phenols, cardiac glycosides, terpenoids and saponins since they have been associated with antioxidant activities.

Data Management and Statistical Analysis

The quantitative data on DPPH scavenging activities, hydrogen peroxide scavenging activities and reducing powers was obtained, entered into broad spreadsheets using Ms excel. The data was expressed as mean ± standard error of the mean (SEM) by descriptive statistics. It was further analyzed by one way- analysis of variance (ANOVA) followed by Tukey's post hoc test. Data from total phenols was analyzed by unpaired t-test. Data was analyzed using Minitab Version 17 statistical software. P ≤ 0.05 was considered statistically significant. To compute the IC₅₀ values, linear regression analysis was used. The qualitative data on phytochemical screening was presented in form of tables.

Results

DPPH Scavenging Activity

The DCM: MeOH leaf extracts of *C. abyssinica* and *M. Obscura* showed remarkable *in vitro* DPPH scavenging activities that increased with increasing extract

concentration (Table 1). At concentrations of 0.0625, 0.125, 0.25 and 1 mg/ml, the DPPH scavenging activity of the DCM: MeOH leaf extracts of *M. obscura* was significantly higher than that of *C. abyssinica* leaf extracts (p<0.05; Table 1).

However, at the extract concentration of 0.5 mg/ml, *M. obscura* was as effective as *C. abyssinica* (p>0.05); Table 1). At all the tested concentrations, the *in vitro* DPPH radical scavenging activity of the standard (ascorbic acid) was significantly higher than that of the two studied plant extracts (p<0.05; Table 1). The IC₅₀ values for the DCM: MeOH extracts of *C. abyssinica*, *M. obscura* and the standard were 0.087, 0.065 and 0.044 respectively (Table 1).

Total Ferric Reducing Power

The DCM: MeOH leaf extracts of *C. abyssinica* and *M. obscura* demonstrated a dose dependent increase in total ferric reducing powers (Table 2). The DCM: MeOH leaf extracts of *M. Obscura* largely produced significantly higher total ferric reducing power than *C. abyssinica* (p<0.05; Table 2). However, at extract concentration of 0.6 mg/ml, both plant extracts were equally effective (p>0.05; Table 2). The standard (ascorbic acid) exhibited significantly higher total ferric reducing power than both plant extracts at all the tested concentrations (p<0.05; Table 2).

Hydrogen Peroxide Scavenging Activity

The DCM: MeOH leaf extracts of *C. abyssinica* and *M. obscura* exhibited hydrogen peroxide scavenging activities that decreased with increasing extracts concentration (Table 3). At all the tested extract concentrations, the DCM: MeOH leaf extracts of *M. obscura* was significantly more effective than the DCM: MeOH leaf extracts of *C. abyssinica* (p<0.05; Table 3).

However, the effects of the two plant extracts were significantly lower than that of the standard (ascorbic acid) at all the tested concentrations (p<0.05; Table 3). The IC₅₀ values for the DCM: MeOH leaf extracts of *C. abyssinica*, *M. obscura* and ascorbic acid were 0.0350, 0.0446 and 0.1104 respectively (Table 3).

Total Phenolic Contents

The DCM: MeOH leaf extracts of *C. abyssinica* and *M. obscura* demonstrated significant levels of total phenolics as shown in table 4. However, the phenolic content of the DCM: MeOH leaf extracts of *M. obscura* was significantly higher than that of *C. abyssinica* (Table 4).

Qualitative Phytochemical Screening

The qualitative phytochemical screening of DCM: MeOH leaf extracts of *C. abyssinica* showed the presence of phenols, alkaloids, flavonoids, tannins, steroids, terpenoids, cardiac glycosides and saponins, while the leaf extracts of *M. obscura* showed the presence of phenolics, flavonoids, alkaloids, steroids, saponins, terpenoids, cardiac glycosides and tannins (Table 5).

Table 1. In vitro DPPH scavenging activities of DCM: MeOH leaf extracts of *C. abyssinica* and *M. obscura*

Treatment	DPPH scavenging activity (% inhibition)					
	Extract concentration (mg/ml)					
	0.0625	0.125	0.25	0.5	1	IC ₅₀
Ascorbic acid	50.52±0.24 ^a	66.94±0.36 ^a	81.01±0.23 ^a	83.82±0.48 ^a	91.77±0.24 ^a	0.044
DCM: MeOH extract of <i>C. abyssinica</i>	46.91±0.27 ^c	54.31±0.37 ^c	60.77±0.11 ^c	71.32±0.25 ^b	80.00±0.11 ^c	0.087
DCM: MeOH extract of <i>M. obscura</i>	49.33±0.16 ^b	58.90±0.27 ^b	64.59±0.30 ^b	72.45±0.22 ^b	82.57±0.16 ^b	0.065

Values expressed as Mean ± SEM of the experiment triplicates in each concentration. Values with the same superscript letters are not significantly different by one way ANOVA followed by Tukey's post hoc test (p>0.05)

Table 2. Total ferric reducing power of DCM: MeOH leaf extracts of *C. abyssinica* and *M. obscura*

Treatment	Absorbance at 700 nm				
	Extract concentration (mg/ml)				
	0.2	0.4	0.6	0.8	1
Ascorbic acid	0.2533±0.005 ^a	0.4130±0.003 ^a	0.5037±0.003 ^a	0.6027±0.004 ^a	0.6207±0.006 ^a
DCM: MeOH extract of <i>C. abyssinica</i>	0.0547±0.003 ^c	0.1067±0.002 ^c	0.1623±0.005 ^b	0.1713±0.004 ^c	0.2177±0.010 ^c
DCM: MeOH extract of <i>M. obscura</i>	0.0917±0.01 ^b	0.1660±0.012 ^b	0.2133±0.023 ^b	0.2877±0.02 ^b	0.3667±0.02 ^b

Values expressed as Mean ± SEM of the experiment triplicates in each concentration. Values with the same superscript letters are not significantly different by one way ANOVA followed by Tukey's post hoc test (p>0.05)

Table 3. Hydrogen peroxide scavenging activities of DCM: MeOH leaf extracts of *C. abyssinica* and *M. obscura*

Treatment	Hydrogen peroxide scavenging activity (%)					
	Extract concentration (mg/ml)					
	0.00625	0.0125	0.025	0.05	0.1	IC ₅₀
Ascorbic acid	90.18±0.28 ^a	83.33±0.28 ^a	73.35±0.09 ^a	64.11±0.18 ^a	56.58±0.16 ^a	0.1104
DCM: MeOH extract of <i>C. abyssinica</i>	75.50±0.30 ^c	68.03±0.27 ^c	54.34±0.23 ^c	42.01±0.33 ^c	36.99±0.16 ^c	0.0350
DCM: MeOH extract of <i>M. obscura</i>	79.73±0.38 ^b	70.69±0.18 ^b	57.16±0.32 ^b	46.03±0.41 ^b	39.92±0.26 ^b	0.0446

Values expressed as Mean ± SEM of the experiment triplicates in each concentration. Values with the same superscript letters are not significantly different by one way ANOVA followed by Tukey's post hoc test (p>0.05)

Table 4. Total phenolic contents of DCM: MeOH leaf extracts of *C. abyssinica* and *M. obscura*

Sample	TPC (mg GAE/g)
<i>Maytenus obscura</i>	78.38±1.03 ^a
<i>Clusia abyssinica</i>	19.82±0.597 ^b

Values expressed as mean ± SEM of the experiment triplicates. Values with different superscript letters are significantly different by unpaired student t-test (P<0.05).

Table 5. Qualitative phytochemical composition of DCM: MeOH leaf extracts of *C. abyssinica* and *M. obscura*

Phytochemical	DCM: MeOH leaf extract of <i>M. obscura</i>	DCM: MeOH leaf extract of <i>C. abyssinica</i>
Phenols	+	+
Flavonoids	+	+
Alkaloids	+	+
Phlobatanins	-	-
Tannins	+	+
Saponins	+	+
Terpenoids	+	+
Steroids	+	+
Cardiac glycosides	+	+

A positive sign (+) denotes presence and negative sign (-) denotes absence of that given phytochemical.

DISCUSSION

Generally, the DCM: MeOH leaf extracts of *C. abyssinica* and *M. obscura* demonstrated significant DPPH free radical scavenging activities in dose dependent fashions (Table 1). These findings were similar to work carried out by [24], where they reported an increase in the DPPH scavenging effects of petroleum ether fruit extracts of *Dregea volubilis* with increase in extract concentration. Another study carried out by [25] showed that the methanolic leaf extracts of *Zanthoxylum armatum* demonstrated a dose dependent increase in DPPH scavenging activity. Studies also reported a dose dependent increase in the DPPH scavenging effects of aqueous bark extracts of *Strychnos henningsii* Gilg. This implies that higher concentrations are associated with stronger free radical scavenging potential [25].

The IC₅₀ values for the DCM: MeOH extracts of *C. abyssinica*, *M. obscura* and the standard (ascorbic acid) DPPH scavenging activities were 0.087, 0.065 and 0.044 respectively (Table 1). The DCM: MeOH leaf extracts of *M. obscura* showed a slightly lower IC₅₀ value than the DCM: MeOH leaf extracts of *C. abyssinica*. This means that a lower concentration of *M. obscura* was needed to cause DPPH scavenging activity than *C. abyssinica*, which would require a slightly higher concentration.

In this study, total ferric reducing power assay for the DCM: MeOH leaf extracts of *C. abyssinica* and *M. obscura* was also done. It is a method based on the principle of reduction of Potassium ferricyanide (Fe³⁺) to Potassium ferrocyanide (Fe²⁺), which reacts with Iron (III) Chloride reducing it to Iron (II) chloride. Reduction of iron (III) Chloride to iron (II) chloride in a reaction mixture is an indicator of an electron-donation activity [26]. The formation of Iron (II) complex was then monitored at 700 nm. Increasing absorbance indicated increase in reducing power. Reducing power is used as an indicator of antioxidant activity [27].

The DCM: MeOH leaf extracts of *C. abyssinica* and *M. obscura* produced dose dependent increase in ferric reducing power. Studies by [28] indicated that the ferric reducing power of different solvent extracts of *Eicnornia crassipes* (mart.) solms increased with increase in extract concentration. Besides, [29] observed a dose dependent increase in ferric reducing power of acetone and aqueous extracts of *Bulbine abyssinica* while determining its antioxidant activities. This implies that as the extract concentration increases, the amount of antioxidant compounds present in the extract increases, thus the increase in ferric reducing power, which shows an increase in antioxidant potential.

Hydrogen peroxide scavenging assay is an assay based on the ability of the extracts to scavenge the hydrogen peroxide and reduce it to water. In this study, it was observed that the DCM: MeOH leaf extracts of *C. abyssinica* and *M. obscura* demonstrated significant

hydrogen peroxide scavenging activities that decreased with increase in concentration (Table 3). A dose dependent decrease in hydrogen peroxide scavenging activity was also demonstrated by both acetone and aqueous whole plant extracts of *Bulbine abyssinica* [29]. However, in a study carried out by [30], hexane extracts of *Cyperus esculentus* produced a dose dependent increase in hydrogen peroxide scavenging activities, while its methanolic extract produced a dose dependent decrease in hydrogen peroxide scavenging activity. Moreover, [31] found that the hydrogen peroxide scavenging effects of cocoa oil and cake increased with increase in concentration. Dose-dependent decrease in hydrogen scavenging activity implies that low concentrations had high scavenging activities hence high antioxidant abilities.

This trend in the hydrogen peroxide scavenging activity could have been due to saturation of reactive centers of hydrogen peroxide by the high extract concentrations leading to low activities, compared to dilute concentrations that ensure easier and rapid reaction, leading to high activity.

The IC₅₀ values for the DCM: MeOH extracts of *C. abyssinica*, *M. obscura* and the standard (ascorbic acid) were 0.0350, 0.0446 and 0.1104 respectively. This means that there was a negative relationship between the concentration and the potential of the hydrogen peroxide scavenging activity.

As shown in table 4, the DCM: MeOH leaf extracts of *C. abyssinica* and *M. obscura* demonstrated a significant amount of total phenolic content (Table 4). However, the phenolic content of the DCM: MeOH leaf extracts of *M. obscura* was significantly higher than that of *C. abyssinica* (Table 4). This explains why the tested antioxidant activities of DCM: MeOH leaf extracts of *M. obscura* were consistently higher than those of DCM: MeOH leaf extracts of *C. abyssinica*.

The observed *in vitro* antioxidant activities of the two studied plant extracts could have been due to the presence of different bioactive secondary metabolites. The qualitative phytochemical screening of DCM: MeOH leaf extracts of *C. abyssinica* showed the presence of phenols, alkaloids, flavonoids, tannins, steroids, terpenoids, cardiac glycosides and saponins, while the leaf extracts of *M. obscura* showed the presence of phenolics, flavonoids, alkaloids, steroids, saponins, terpenoids, cardiac glycosides and tannins (Table 5).

Phenols have been linked to antioxidant activity due to their ability to quench and neutralize free radicals by donation of hydrogen atom or electrons [24]. A study by (1) linked the strong antioxidant activity of *Dicliptera roxburghiana* to the high total phenolic content of the plant extract.

Flavonoids have the ability to act as free radicals scavengers, suppressors of the activities of free radicals production enzymes and stimulators of antioxidant

enzymes. They can also act as electron donors and metal chelating molecules [4, 24, 30] Studies by [7] attributed the antioxidant activities of *Viciacaescece* to its flavonoid constituents.

Tannins act as antioxidants by donating hydrogen atom or electrons to other molecules thus stabilizing them. They also act as metal chelators [2]. In a study by [32], tannins have been linked to the antioxidant activity of *Passiofiora foetida* L. In different *in vitro* assays, tannic acid was found to be an effective antioxidant molecule [9].

CONCLUSION

The results of the this study demonstrated that the DCM: MeOH leaf extracts of *C. abyssinica* and *M. obscura* exhibited remarkable scavenging effects on

DPPH radical, total ferric reducing effects and hydrogen peroxide scavenging activities. This study therefore validates the use of the two plants in the management of oxidative stress. They can serve as alternatives to conventional ways of managing oxidative stress-related disease conditions.

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CONFLICT OF INTEREST

The author (s) declares that there is no conflict of interest regarding the publication of this article.

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