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Assessment of 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging, total antioxidant activity, ferric reducing power and phytochemical analysis of methanolic extract of *Malva parviflora*

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Abstract

In this study, preliminary phytochemical screening and *in vitro* antioxidant activity of crude methanolic extract of whole plant of *Malva parviflora* were evaluated. Phytochemical screening was achieved using standard assay methods and for antioxidant activity, three assays viz. 2,2-diphenyl-1-picrylhydrazyl (DPPH) assay, phosphomolybdate assay (total antioxidant capacity, TAC) and ferric reducing power assay were used to screen the extract at various concentrations. The extract demonstrated the presence of alkaloids, flavonoids, phenolics, tannins and terpenoids. The DPPH radical scavenging activity and total antioxidant capacity of methanolic *M. parviflora* extract (MMP) were found to be in the range 41.88±1.69 to 95.44±2.91% and 52.45±1.45 to 89.65±2.84% respectively. MMP was found to be potent with IC_{50} values for DPPH and TAC being 91.61 and 29.15 µg/mL, respectively. MMP was found to be dose-dependent as its reducing power increased with extract concentration. Generally, MMP exhibited promising phytochemical and antioxidative properties as a potential source for novel drugs. As such, further studies are required on this plant in order to commercial new products.

Keywords: Phytochemical screening, DPPH assay, phosphomolybdate assay, methanol extract, *Malva parviflora*

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1. Introduction

The use of herbal medicine to treat various symptoms of diseases dates back to very ancient times and still finds a wide application up to date despite recent advances in medicine (Saeed *et al.,* 2012). Such a wide application of herbal medicine especially in developing countries stems from their prophylactic abilities and their long line of application as folk medicine (Saeed *et al.,* 2012). Medicinal plants have been found to contain natural antioxidants which effectively prevent disruptive effects of oxidative stress (Zengin *et al.,* 2011). In recent years, much attention has been drawn to antioxidants derived from natural sources owing to their significant free radical scavenging properties (Osawa *et al.,* 1990).

Known locally as "tika-motse" and commonly by other names such as cheese weed, smallwhorl mallow, common mallow and cheeseweed mallow, *Malva parviflora* L. belongs to the Malvaceae family of the Malva genus (Singh *et al.,* 2017; Aslam *et al.,* 2014). *M. parviflora* is a herbaceous, perennial plant which grows up to 1.2 meter height and potential width of 2.1 meter (Singh and Navneet, 2017; Coleman *et al.,* 2019). It possesses 5 to 7 toothed, scalloped lobes leaves with a dark-green appearance and also has a woody base that is strongly tap-rooted (Coleman *et al.,* 2019; Afolayan *et al.,* 2008). *M. parviflora* is distinguished by its clustered, white to dusty pink flowers with penta-notched petals (Singh and Navneet, 2017). It has no favourable soil media for growth (Coleman *et al.,* 2019). *M. parviflora* has been used in the mountain kingdom of Lesotho and many parts of the world to alleviate various symptoms of diseases. Whole plant of *M. parviflora* has been used to treat pain, inflammation, abscesses, liver injuries, swellings, boils and soften tumors (Singh and Navneet, 2017; Altyar *et al.,* 2022; Islam *et al.,* 2010; Afolayan *et al.,* 2010). Tea prepared from the leaves of *M. parviflora* is

used to alleviate dry coughs, bronchitis, profuse menstruation and also applied as taenicide (Singh *et al.,* 2017; Ishtiaq *et al.,* 2012). Lotion prepared from *M. parviflora* leaves is used to treat broken limbs, bruises and as antidandruff (Islam *et al.,* 2010; Shale *et al.,* 1999). Infusions of leaves and roots are used in Lesotho to clean sores (Shale *et al.,* 1999). Ethanolic extracts of
M. parviflora species growing in Lebanon have been found to exhibit significant antioxidant activity when challenged using three different *in vitro* methods, namely; DPPH, hydrogen peroxide and chelating ferrous ions tests (Farhan *et al.,* 2012). *In vivo* antifungal and antioxidant activity of ethanolic extract of *Malva parviflora* has been studied as well in male rats (Muhammed Ridh *et al.,* 2018). Anti-inflammatory, analgesic, antibacterial, antidiabetic, anti-irritant, neuroprotective, hepatoprotective and antifungal activities from this plant have also been previously reported (Singh *et al.,* 2017; Aslam *et al.,* 2014; Islam *et al.,* 2010; Afolayan *et al.,* 2010; Shale *et al.,* 1999).

To the best of our knowledge, phytochemical and antioxidant properties of *M. parviflora* species growing in the Kingdom of Lesotho have not been largely explored. The aim of the present study was to evaluate *in vitro* antioxidant activity of crude methanolic extract of *M. parviflora* and screen the extract for phytoconstituents. The results are communicated in this article.

2. MATERIALS AND METHODS

2.1. Chemicals and reagents

Methanol (AR Grade, 99.5%), trichloroacetic acid, Sulphuric acid (98%) and 2,2-diphenyl-1- picrylhydrazyl (DPPH) were purchased from Sigma Aldrich. L(+)-ascorbic acid, tri-sodium orthophosphate (Na_3PO_4) and ferric chloride ($FeCl_3$) were purchased from Associated Chemicals Enterprise. Hydrochloric acid (32%), Sodium dihydrogen phosphate (NaH_2PO_4), Sodium hydrogen phosphate (Na_2HPO_4) and potassium ferricyanide ($K_3[FeCN_6]$) were purchased from Prestige Laboratory Supplies in Republic of South Africa.

2.2. Plant materials

Fresh plant materials of *M. parviflora* were collected in October 2022 inside Roma campus of National University of Lesotho. The plant species was identified and authenticated at the herbarium of the Department of Biology and Biotechnology, National University of Lesotho. A voucher specimen viz. Polile /RP/2022 was deposited at the Pharmaceutical Chemistry Research Laboratory, National University of Lesotho, Roma Campus.

2.3. Processing of plant materials

Whole plant materials were dried in the shade at room temperature for 11 days. The air-dried whole plant materials were then crushed into powder using a laboratory blender (Waring Blender, Blender 80119, Model HGB2T93, 240V, 3.5 AMPs). 87.5362g powdered sample was obtained.

2.4. Preparation of plant extracts

A mass of 87.5362g powdered sample was macerated with 200mL methanol for three days. The solution was filtered off using a vacuum pump. The extract was concentrated in *vacuo*. The extract was then collected in a pre-weighted clean and dry beaker. The exact procedure was performed twice. 9.0561g combined methanolic extract was obtained.

2.5. Antioxidant assay

The methanolic extract was dissolved in absolute methanol to make a stock solution with concentration of 1000 µg/mL. From the stock solution, two-fold serial dilutions of concentrations of 1000, 500, 250, 125, 62.5 and 31.25 µg/mL were prepared for DPPH and phosphomolybdate antioxidant assays (Kheoane and Mokonyana, 2022). For FRAP assay, two-fold serial dilutions of 200, 100, 50, 25 and 12.5 µg/mL were prepared. The 0 µg/mL solution served as the negative control and ascorbic acid was maintained as the positive control in all the assays.

2.5.1. DPPH assay

The free radical scavenging activity of crude methanolic extract of *M. parviflora* was measured *in vitro* by DPPH assay as described in literature (Kheoane and Mokonyana, 2022; Hsu et al., 2006), though with modifications. A solution composed of 100 µM DPPH in methanol was prepared. 500 µL of the previously prepared plant extract solution was mixed with 2.0 mL of 100 µM DPPH in a 20 mL test tube. The contents were vortexed and

incubated in a dark cupboard for 30 min to minimize degradation. Optical density of the previously incubated mixture and control solutions was taken at 517.0 nm by MRS Spectro UV – 11 spectrophotometer. As mentioned earlier, ascorbic acid served as the positive control and methanol was used as the negative control. Percentage inhibition of DPPH free radical was determined using the following equation:

Scavenging % = [(Ac – As)/Ac] x 100

Where Ac and As denote absorbance at 517.0 nm of mixture without sample and with samples respectively (Pathak *et al.,* 2013).

IC_{50}, the concentration of the extract that yield 50% inhibition, was generated by Microsoft Excel (Risdian *et al.,* 2011).

2.5.2. *Phosphomolybdate assay* (*Total antioxidant capacity*)

The total antioxidant capacity (TAC) of the crude methanolic extract of *M. parviflora* was measured using phosphomolybdate method as described in literature (Adewusi *et al.,* 2011) but with some modifications. 100 µL of the plant extract solution was mixed with 1.0 mL phosphomolybdate reagent (28 mM tri-sodium phosphate, 4 mM ammonium molybdate and 0.6 M sulphuric acid) in test tubes. The test tubes were capped with aluminum foil and incubated in water bath maintained at 95 ℃ for 90 min. At the end of the incubation period, the contents were cooled to room temperature and the absorbances were taken at 765.0 nm by MRS Spectro UV – 11 spectrophotometer. Similarly, solution without the extract served as blank and that with ascorbic acid served as positive control. The antioxidant effect was calculated using the following equation (Zengin *et al.,* 2011).

Antioxidant effect (%) = [(Control absorbance – Sample absorbance)/control absorbance] x 100

The IC_{50} value of the methanolic extract was also generated using Microsoft Excel.

2.5.3. Evaluation of ferric reducing ability

The method adopted by Fejes *et al*. (2000) was used to screen the extract for its reducing capacity. This method is founded on the ability of the extract to reduce Fe (III) to Fe (II) (Umamaheswari and Chatterjee 2008). This can be tracked by measuring the formation of Prussian blue colour at 700.0 nm, indicating the formation of Fe (II). Aliquots of 2.0 mL of the plant extract at various concentrations were mixed separately with 2.0 mL of 0.2 M phosphate buffer (pH 6.6) and 2.0 mL of 10 g/L potassium ferricyanide. The mixture was incubated at 50℃ for 20 min and 2.0 mL of 0.1 g/L trichloroacetic acid was added thereafter. The contents were then centrifuged at 3000 rpm for 10 min to abstract the upper layer. Aliquots of 2.0 mL of the supernatant solution were further mixed separately with 400 µL of 0.1% (w/v) freshly prepared iron (III) chloride and 2.0 mL distilled water. The absorbance of the contents was then taken at 700.0 nm after the 10-minute reaction. The higher the absorbance of the reaction mixture, the higher the reducing power of the extract (Umamaheswari and Chatterjee 2008).

2.6. Phytochemical screening

Pre-phytochemical screening of methanolic *M. parviflora* extract (MMP) for the presence of alkaloids, flavonoids, phenolics, tannins and terpenoids was performed.

2.6.1. Test for alkaloids

The method adopted by Harbone (1973) was employed to carry out this test (Coleman *et al.,* 2019). An amount of 0.4g of MMP and 8mL of 1% HCl were mixed and stirred. The resulting mixture was warmed and filtered (Coleman *et al.,* 2019). Aliquots of 2.0 ml of the filtrate were mixed separately with few drops of Mayer's reagent (potassium mercuric iodide). The formation of a precipitate or turbidity indicated the presence of alkaloids.

2.6.2. Test for flavonoids

The method adopted by Saeed *et al.* (2017) was employed to conduct this test (Zengin *et al.,* 2011). A volume of 10.0 mL of the filtrate abstracted from 50mg of MMP suspended in 100 mL of distilled water, was mixed with 5.0 mL of dilute ammonia solution. This step was followed by addition of few drops of concentrated sulphuric acid. The formation of a yellowish colouration demonstrates the presence of flavonoids (Hossain *et al.,* 2011).

2.6.3. Test for phenolics and tannins

The method adopted by Saeed *et al*. 2017 was used to carry out this test (Zengin *et al.,* 2011). An amount of 50mg of MMP was mixed with 20.0 mL of distilled water then boiled and filtered to obtain the resulting filtrate. The brownish green or blue-black colouration upon addition of few drops of 0.1% (w/v) ferric chloride was considered as the positive test for the presence of phenolics and tannins (Farhan *et al.,* 2012).

2.6.4. Test for terpenoids

The method adopted by Harbone (1973) was used to perform this test (Coleman *et al.,* 2019). A volume of 5.0 mL of 1.0 mg/mL MMP was mixed with 2.0 mL of chloroform. The formation of brown-red colour on the interface upon the addition of 3.0 mL of concentrated sulphuric acid to the mixture verified the presence of terpenoids.

2.7. Statistical analysis

All determinations were performed in triplicates and the results were expressed as mean±standard deviation. Statistical significance was achieved using one-way analysis of variance (ANOVA) at $p<0.05$.

3. RESULTS AND DISCUSSION

3.1. Phytochemical screening

Table **1** summarizes the preliminary phytochemical screening profile of methanolic *M. parviflora* extract (MMP). MMP exhibited the presence of alkaloids, flavonoids, phenolics, tannins and terpenoids.

Table 1: Qualitative phytochemical screening of methanolic extract of *M. parviflora*

No.	Tests conducted for phytoconstituents	Solvent extract
		Methanol
1	Flavonoids	+
2	Phenols (Ferric chloride test)	+
3	Terpenoids (Salkwoski's test)	+
4	Alkaloids (Mayer and Wagner's test)	+
5	Tannins (Ferric chloride test)	+
	Total	5

Presence of the phytoconstituents is denoted by (+) and the (-) sign for showing absence

It has been previously reported that, the leaves and stems of *M. parviflora* species growing in Lebanon contain polyphenols, tannins, flavonoids, resins, alkaloids and saponins (Hossain *et al.,* 2011). The phytochemical screening results obtained in our study corresponds to the findings in Farhan *et al*. (2012) report.

3.2. DPPH radical scavenging activity

Table **2** summarizes the inhibition percentage of DPPH radical and phosphomolybdate by the crude methanolic extract and ascorbic acid. MMP exhibited DPPH radical scavenging activity of 41.88±1.69, 43.14±4.07, 57.93±1.19, 75.30±6.86, 81.39±2.04 and 95.44±2.91% at concentrations of 31.25, 62.5, 125, 250, 500 and 1000 µg/mL, respectively. On the other hand, the positive control exhibited scavenging activity of 46.74±0.64, 48.41±3.67, 62.02±1.82, 74.61±0.76, 84.54±1.37 and 98.52±1.93% at similar concentrations of 31.25, 62.5, 125, 250, 500 and 1000 µg/mL, respectively.

Table 2: percentage inhibition of antioxidant assays by the methanolic extract and ascorbic acid

Concentration	%DPPH inhibition		%Phosphomolybdate inhibition	
(µg/mL)	MMP	Asc. acid	MMP	Asc. Acid
31.25	41.88±1.69	46.74±0.64	52.45±1.45	54.26±2.77
62.5	43.14±4.07	48.41±3.67	57.38±0.99	54.81±5.30
125	57.93±1.19	62.02±1.82	65.26±3.01	76.74±0.47
250	75.30±6.86	74.61±0.76	69.33±1.16	89.28±2.68
500	81.39±2.04	84.54±1.37	78.62±1.14	97.17±1.96
1000	95.44±2.91	98.52±1.93	89.65±2.84	100.96±4.31

MMP = methanolic *M. parviflora* extract, Asc. acid = Ascorbic acid which served as positive control. The experiments were carried out in triplicates and each value was expressed as mean±SD (n=3)

This result showed that, at low concentrations of 31.25 and 62.5 µg/mL, MMP has a weak radical scavenging potential but at concentrations of 125 µg/mL and above, MMP has comparatively high radical scavenging activity relative to the positive control. Table **3** also summarized the IC_{50} values for DPPH radical scavenging potential of MMP and ascorbic acid. MMP exhibited IC_{50} value of 92.61 µg/mL while the positive control exhibited 68.46 µg/mL. This result shows that MMP has a promising anti-radical potential relative to ascorbic acid.

DPPH radical scavenging activity and ferrous reducing antioxidant power (FRAP) of ethanolic and water extracts of *M. parviflora* leaves and stems have previously been reported [14]. Ethanolic leaves and stem-bark extracts exhibited IC_{50} values of 1.0 and 1.9 mg/mL, respectively, whereas water leaves and stem-bark extracts exhibited IC_{50} values of 1.0 and 1.5 mg/mL respectively, for DPPH radical scavenging test (Farhan *et al.,* 2012). Leaves and stem extracts from Lebanese *M. parviflora* have been found to exhibit DPPH IC_{50} values of 2.52 and
2.74 mg/mL respectively (Hossain *et al.,* 2011). The methanolic and aqueous leaves extract of
M. parviflora species collected in Algeria have been found to exhibit IC_{50} values of 89.03±2.65 and 76.67±0.29 mg/mL, respectively, under DPPH test (Abdel-Ghani *et al.,* 2011). The methanol extract is common to our study, however, we evaluated a whole plant extract and in their case the leaves extract. In our study, the methanolic extract of *M. parviflora* showed an IC_{50} value of 92.61 µg/mL whereas in the case of Bouriche *et al.,* 2011, the methanolic leaves extract was found to be much more potent with IC_{50} value of 21.05 µg/mL (Abdel-Ghani *et al.,* 2011).

3.3. Total antioxidant activity

Table **2** also condensed the total antioxidant capacity of MMP and ascorbic acid. MMP exhibited the total antioxidant capacity of 52.45±1.45, 57.38±0.99, 65.26±3.01, 69.33±1.16,
78.62±1.14 and 89.65±2.84% at concentrations of 31.25, 62.5, 125, 250, 500 and 1000 µg/mL, respectively. The positive control exhibited total antioxidant capacity of 54.26±2.77, 54.81±5.30, 76.74±0.47, 89.28±2.68, 97.17±1.96 and 100.96±4.31% at similar concentrations
of 31.25, 62.5, 125, 250, 500 and 1000 µg/mL, respectively. This result showed that MMP has comparable antioxidant effect relative to the positive control at all corresponding concentrations.

Table 3: IC_{50} values for DPPH and TAC of methanolic *M. parviflora* extract and ascorbic acid

Extract	**DPPH: IC50 (µg/mL)**	**TAC: IC50 (µg/mL)**
MMP	92.61	29.15
Asc. Acid	68.46	21.05

Table **3** also summarizes the IC_{50} values for TAC of MMP and ascorbic acid. MMP exhibited IC_{50} value of 29.15 µg/mL whereas the positive control exhibited 21.05 µg/mL. This result showed that MMP has a comparable potency relative to the positive control.

There is no comparable literature for whole plant extract of *M. parviflora*.

3.4. Ferric reducing ability

Table **4** and figure **1** both depict the absorbance of various concentrations of the methanolic crude extract showing their reducing capacity. The results showed that the reducing ability of MMP increased with extract concentration.

Table 4: Reducing power of crude methanolic extract of *M. parviflora* and ascorbic acid

Concentration (µg/mL)	Absorbance	
	MMP	Asc. Acid
12.5	0.155±0.005	0.164±0.070
25	0.199±0.018	0.279±0.006
50	0.217±0.056	0.599±0.099
100	0.759±0.008	1.167±0.023
200	0.804±0.102	1.793±0.002

Ethanolic leaves and stem-bark extracts exhibited IC_{50} values of 3.8 and 4.5 mg/mL, respectively, whereas water leaves and stem-bark extracts exhibited IC_{50} values of 3.0 and 3.9 mg/mL respectively, for FRAP test (Farhan *et al*., 2012).

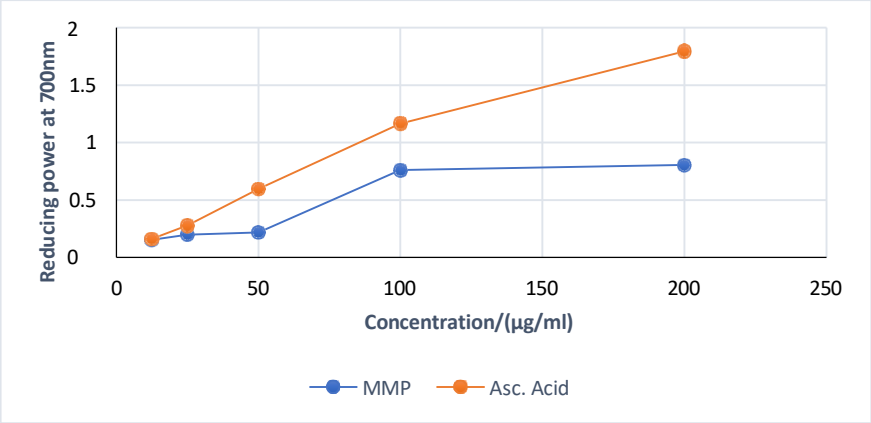

Figure 1: Reducing capacity of MMP and ascorbic acid.

Crude ethanolic extract of *M. parviflora* collected in Egypt has been reported to exhibit potent cytotoxic potential with IC_{50} value of 0.87 µg/w (Jaradat *et al.,* 2015). Additionally, the methanolic and aqueous leaves collected in Lebanon were challenged for their ferrous chelating potential and they were found to exhibit IC_{50} values of 349.71±7.25 and 42.26±0.61 mg/Ml (Abdel-Ghani *et al.,* 2011). Tannins have been reported to have ferric (Fe^{3+}) reducing abilities and also possess important inflammatory properties due to their ability to impede the 5- lipoxygenase enzyme in the metabolism of arachidonic acid (Okuda, 2005). Our study confirmed the presence of tannins in *M. parviflora* and thus the methanolic extract exhibited such promising reducing ability.

4. Conclusion

We have evaluated the preliminary phytochemical screening and *in vitro* antioxidant activity of the crude methanolic extract of *M. parviflora* collected from the kingdom of Lesotho. The extract demonstrated the presence of the five tested phytoconstituents, namely; alkaloids, flavonoids, phenolics, tannins and terpenoids. The DPPH radical scavenging activity and total antioxidant capacity of *M. parviflora* methanolic extract (MPME) was found to be in the range 41.88±1.69 to 95.44±2.91% and 52.45±1.45 to 89.65±2.84% respectively. Crude methanolic extract of *M. parviflora* was found to be potent with DPPH and TAC of IC_{50} values of 91.61 and 29.15 µg/mL, respectively. The reducing power increased with extract concentration. Based on the aforementioned results, we therefore concluded that, *M. parviflora* species from the Kingdom of Lesotho exhibit remarkable antioxidant activities.
Bio-guided fractionation to isolate pure compounds from this plant is required for further studies.

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