

PHYTOCHEMICAL AND FREE RADICAL SCAVENGING ACTIVITY OF HENNA LEAVES EXTRACTS

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ABSTRACT

The ability of *Lawsonia inermis* (Henna) to prevent the initiation of free radicals that cause cellular damage was investigated *in vitro*. The plant leaves were extracted using methanol (ME) and diethylether (EE) and the active ingredient, Lawsone (2-hydroxy 1,4-naphthaquinone) was separated by column chromatography. In the reducing power assay, the more antioxidant compounds convert the oxidation form of iron (Fe^{+3}) in ferric to ferrous chloride (Fe^{+2}). The reducing power capacity of the methanol and etheric extracts were significantly less than that of vitamin C. Total phenolic (TPC) and total flavonoid contents (TFC) were evaluated. In this study, etheric extract has the highest TPC and TFC. Radical scavenging activities of lawsone and the extracts were tested against DPPH, NO^{\cdot} , SOD and HO^{\cdot} . The strongest free radical inhibition was demonstrated by etheric extract against radical scavengers, DPPH and SOD in a dose-dependent on IC_{50} value of 18.17 and 137 $\mu g/mL$, respectively. Also, the activity of lawsone to scavenge nitric oxide radical was higher than that of the standard (quercetin) and (ME, EE) extracts. These results clearly indicate the useful effect of lawsone and these extracts as antioxidants and anticancer agents.

Keywords: *Lawsonia inermis*, Free radicals, Antioxidant, Lawsone, Radical scavengers.

INTRODUCTION

Free radicals are created when cells use oxygen to generate energy. These by-products are generally reactive oxygen species (ROS) such as superoxide anion, hydroxyl radical and hydrogen peroxide that result from the cellular redox process.

At low or moderate concentrations, ROS exert beneficial effects on cellular responses and immune function but at high levels, free radicals and oxidants generate oxidative stress, a deleterious process that can damage cell structures, including lipids, proteins, and DNA (Pham-Huy *et al.*, 2008). Oxidative stress plays a major part in the development of chronic and degenerative ailments such as cancer, autoimmune disorders, rheumatoid arthritis, cataract, aging, cardiovascular and neurodegenerative diseases, (Willcox *et al.*, 2004 and Pham-Huy *et al.*, 2008). The human body has several mechanisms to immune oxidative stress by producing antioxidants, which are either naturally produced *in situ*, or externally supplied through foods and/or supplements. These antioxidants act as free radical scavengers

by preventing and repairing damages caused by ROS, and therefore can enhance the immune defense and lower the risk of cancer and degenerative diseases (Pham-Huy *et al.*, 2008). In recent years, there is an increasing interest in finding antioxidant phytochemicals, because they can inhibit the propagation of free radical reactions, protect the human body from diseases (Terao and Piskula, 1997). The most effective components seem to be flavonoids and phenolic compounds of many plant raw materials, are found particularly in herbs, seeds, and fruits. Their metal-chelating capabilities and radical-scavenging properties have enabled phenolic compounds to be as effective free radical scavengers and inhibitors of lipid peroxidation (Terao and Piskula, 1997).

Henna (*Lawsonia inermis* Linn.) invites attention of the researchers worldwide for its important pharmacological activities as anti-inflammatory and anticancer agents. *Lawsonia inermis* Linn. (Family: Lythraceae) is a much branched glabrous shrub or small tree (2 - 6 m in height), cultivated for its leaves although stem bark, roots, flowers and seeds have also been used in traditional medicine. This plant is a worldwide known as a cosmetic agent used to stain hair, skin and nails (Hanna *et al.*, 1998). The plant is reported to contain 14 active components i.e. Lawsone, Esculetin, Fraxetin, Isoplumbagin, Scopoletin, Betulin, Betulinic acid, Hennadiol, Lupeol, Lacoumarin, Laxanthone, Flavone glycosides and two pentacyclic triterpenes. It has analgesic, hypoglycemic, hepatoprotective, immunostimulant, anti-inflammatory, antibacterial, wound healing, antimicrobial, antifungal, antiviral, antiparasitic, antitrypanosomal, antidermatophytic, antioxidant, antifertility, tuberculostatic and anticancer properties, (Chaudhary *et al.*, 2010).

The present study on henna leaf extracts and its active constituent, lawsone was carried out to evaluate the phytochemical and antioxidant effect.

MATERIALS AND METHODS

Collection of Plant And Authentication

The leaves of *Lawsonia inermis* were collected from gardens of New Damietta in August 2012 and the plant materials were authenticated by Prof. M. Serag, Department of Botany, Faculty of Science, Damietta University.

Extraction

Methanolic Extract (ME)

Powder of dried henna leaves (40 g) were mixed with methanol (1.0 L) in a closed flask and kept for 24 hrs. Shaking frequently during the first 6 hours and allowed to stand for 18 hours. Thereafter, it was filtered rapidly taking precautions against the loss of the solvent. The methanolic extract was concentrated to dryness in a rotary evaporator under reduced pressure and controlled temperature (40 - 50 °C). The extract was stored in a refrigerator at 4 °C till further use.

Etheric Extract (EE)

The etheric extract method according to (Berger and Sicker, 2009) has been followed with some modifications. Powder of dried henna leaves (40 g)

were placed in a large beaker and distilled water (2.0 L) were added. The suspension was stirred using a magnetic stirrer with heating while the temperature is kept at 70 °C. After 45 min, the colour of the green suspension turns to brown. After 4 h, solid NaHCO₃ (8.4 g) is added. The suspension is filtered by gravity overnight over three large glass funnels with filter paper (diameter 30 cm). The filtrates are combined and acidified to pH 3.0 using 0.12 M HCl and extracted with diethyl ether (4 × 200 mL). The combined ethereal phases were washed with water (3 × 50 mL) and dried over anhydrous Na₂SO₄, then concentrated to dryness in a rotary evaporator and gave a crude, 0.6 g, which kept in a refrigerator at 4 °C till further use.

Separation of Lawsone (L)

The above crude lawsone was purified by column chromatography. The yield of pure lawsone was 10 % of the crude which checked by TLC (0.06 g, m.p. 191-193 °C) and had similar IR spectroscopy typical to that of the authentic commercial sample of 2-hydroxy-1,4-naphthaquinone which purchased from Sigma-Aldrich company, Germany.

Phytochemical Screening

Terpenes, tannins, flavonoids, saponins, resins and alkaloids were detected in methanolic extract according to the method described by Harborne (1998).

Determination of Total Phenolic Content

Total polyphenols were determined using the Folin-Ciocalteu method according to (Li *et al.*, 2007). The absorbance was measured at 750 nm. using gallic acid (10 – 200 mg/mL), a standard curve was obtained with linearity. Total phenol contents were expressed as milligram gallic acid equivalent (GAE)/g extract.

Determination of Total Flavonoids Content

Aluminum chloride colorimetric method was used for the determination of total flavonoids as described in the method of Chang *et al.*, 2002, using quercetin as standard. The calibration curve was prepared using quercetin solutions of 2.5, 10, 15, 20, 25 and 30 µg/mL concentrations in methanol. The flavonoids content was determined from the standard curve and the results were expressed as mg of quercetin equivalents (QE)/g extract.

HPLC For Polyphenols of Plant Leaves Extract

Phenolic compounds were extracted according to the method outlined by Ben-Hammouda *et al.* (1995).

Identification of individual phenolic compound was performed on a Hewlett-Packard HPLC (Model 1100), using a hypersil C18 reversed phase column (250×4.6 mm) with 5 µl particle size. Injection by means of a Rheodyne injection valve (Model 7125) with 50 µm fixed loop was used. A constant flow rate of 1.0 ml/min was used with two mobile phases (A) 0.5 % acetic acid in distilled water at pH 2.6 and (B) 0.5 % acetic acid in 99.5 % acetonitrile. The elution gradient was linear starting with (A) and ending with (B) over 35 minutes, using an UV detector set at wavelength 254 nm. Phenolic compounds of each sample were identified by comparing their relative retention times with those of the standards mixture chromatogram. Ten standard phenolic compounds were used namely pyrogallol, gallic acid,

protocatechuic, catechin, catechol, vanillic acid, salicylic acid, o-coumaric acid, ellagic acid and cinnamic acid, which were obtained from sigma (St. Louis, USA) and Merck-Schuchardt (Munich, Germany) Chemical Companies.

Nitric Oxide Radical Scavenging Activity

The nitric oxide radical scavenging capacity of the plant leaf constituents was measured by Griess reaction (Sangameswaran *et al.*, 2009). Quercitin was used as reference standard and was treated in the same way as that of the sample. Sodium nitroprusside in phosphate buffer saline (PBS) was used as control. The nitric oxide radical scavenging activity of extracts, lawsone and quercitin was calculated according to the following equation:

$$\% \text{ Nitric oxide radicals scavenging} = [(A_0 - A_1) / A_0] \times 100$$

A₀: Absorbance of sodium nitroprusside in PBS (without sample or Quercitin).

A₁: Absorbance in the presence of the sample or quercitin.

Scavenging of Hydroxyl Radical

The hydroxyl radicals (HO[•]) in aqueous media were generated through the Fenton system. The hydroxyl radical bleached the safranin, so decreased the absorbance of the reaction mixture indicating a decrease in hydroxyl radical scavenging ability (Winterbourn and Sutton, 1984). The absorbance was measured at 520 nm. The suppression ratio for HO[•] radical was calculated from the following expression:

$$\% \text{ Hydroxyl Radical Scavenging} = [(A_i - A_0) / (A_c - A_0)] \times 100$$

A_i: Absorbance in the presence of the tested compound.

A₀: Absorbance in the absence of the tested compound.

A_c: Absorbance in the absence of the tested compound, EDTA-Fe(II) and H₂O₂.

Estimation of Reducing Power

Reducing power assay was determined according to the method of (Ferreira *et al.*, 2007). Absorbance was measured at 700 nm against the corresponding blank solution.

DPPH (2, 2 -diphenyl-1-picryl-hydrazyl) Free Radical Scavenging Activity

DPPH radical scavenging capacity was determined according to the technique reported by (Martins *et al.*, 2008). Sample solution was diluted with DMSO and in each reaction mixture, the solution was mixed with 2.0 mL of 100 μM DPPH. The mixture was shaken vigorously and allowed to reach a steady state at room temperature for 30 min. in dark. Decolorization of DPPH was determined by measuring the absorbance at 517 nm.

$$\% \text{ Scavenging Activity} = (A_0 - A_1 / A_0) \times 100$$

A₀: Absorbance without extract.

A₁: is the absorbance in the presence of the extract or standard sample.

Superoxide Dismutase Activity Assay

Superoxide dismutase activity was determined by the method of (Dechatelet *et al.*, 1974). The inhibition percent was calculated from the following equation:

% Inhibition of reduction for nitro blue dye = [(Rate of absorbance change of blank - Rate of absorbance change of extracts) / Rate of absorbance change of blank] X 100.

RESULTS AND DISCUSSION

Phytochemical Screening of Henna

The preliminary phytochemical screening reveals the presence of terpenes, tannins, flavonoids, resins and alkaloids, however saponins is not found in the sample. The results were shown in Table 1.

Table 1. Qualitative analysis of phytochemical constituents of henna leaves

Constituent	Level*
Terpenes	++
Tannin	++
Flavanoid	++
Saponins	—
Resins	+
Alkaloids	++

* + = Low concentration, ++ = High concentration, — = Not detectable.

Quantitative Estimations

Two different solvent extracts were used in this study i.e methanol and diethylether.

The total polyphenols contents (TPC) in henna leaves ranged from 71.16 in methanolic extract to 150.83 mg GAE/g in etheric extract. Phenols and polyphenolic compounds, such as flavonoids, are widely found in food products derived from plant sources, and they have been shown to possess significant antioxidant activities (Nagavani *et al.*, 2010). Total flavonoids content (TFC) of the extracts were also determined using aluminum chloride assay. The total flavonoids content (TFC) varied from 32.91 in methanolic extract to 67.42 mg QE/g in etheric extract. The etheric extract has the highest amounts of TFC (67.42 mg QE/g extract), while the lowest amount (32.91 QE/g extract) was observed in methanolic extract. In this study, etheric extract has the highest TPC and TFC, that can be further tested for its ability to inhibit different free radicals. Total phenolic and flavonoid contents for henna leaves extracts are collected in Table 2.

Table 2. Total phenolic and flavonoid contents for henna leaf extracts

Extracts	Total Polyphenols (mg GAE/g) ^a	Total Flavonoids (mg QE/g) ^b
Methanolic	71.16	32.91
Etheric	150.83	67.42

^a(mg GAE/g): mg of gallic acid equivalent per g of dry plant extract.

^b(mg QE/g): mg of quercetin equivalent per g of dry plant extract.

HPLC For Polyphenols Extracted From Plant Leaves

Figure 1, shows HPLC chromatogram with different retention times and peak area for main components found in henna leaves. Comparing results with standard compounds, it could be noticed that catechol was the predominant identified compounds with a percentage value of 8.05 % as shown in Table 3. Other nine components could be detected and collected in Table 3, which are ellagic acid, salicylic acid, protocatechuic acid, vanillic acid, catechin, gallic acid, o-coumaric acid, pyrogallol and cinnamic acid. The chromatogram also shows a highest area % percent of 13.0 for unknown compounds.

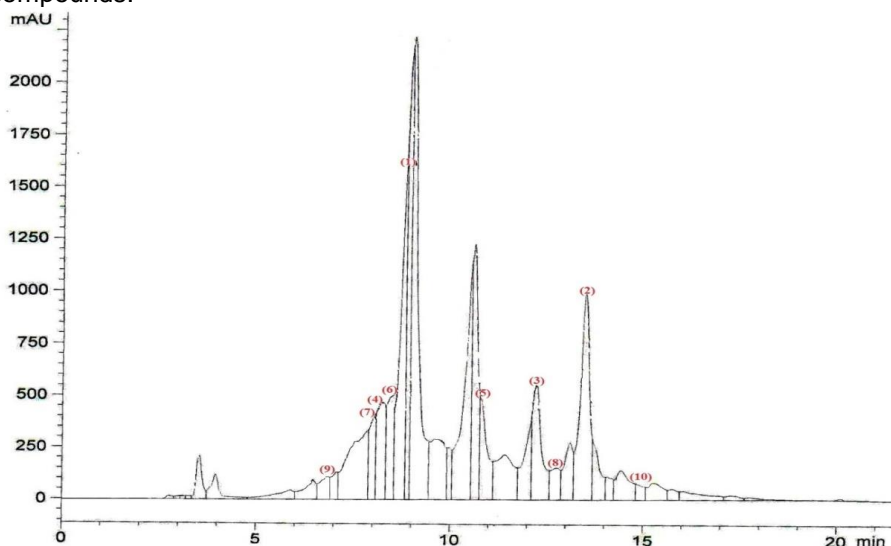


Fig. 1. HPLC chromatogram of *Lawsonia inermis* leaves. (1) Catechol, (2) Ellagic acid, (3) Salicylic acid, (4) Protocatechuic acid, (5) Vanillic acid, (6) Catechin, (7) Gallic acid, (8) o-Coumaric acid, (9) Pyrogallol and (10) Cinnamic acid.

Table 3. Retention time (Rt) and area % for polyphenol components in henna leaves separated by HPLC

No.	Components	Rt	Area %
1	Catechol	8.823	8.047
2	Ellagic acid	13.489	8.039
3	Salicylic acid	12.224	4.484
4	Protocatechuic acid	8.203	3.384
5	Vanillic acid	10.760	3.081
6	Catechin	8.464	3.034
7	Gallic acid	8.067	2.056
8	o-coumaric acid	12.754	1.270
9	Pyrogallol	6.827	0.908
10	Cinnamic acid	14.807	0.516
11	Unknown	8.980	13.00

Antioxidant Activity

The antioxidant reactions involve multiple steps including the initiation, propagation, branching and termination of free radicals. The antioxidants which inhibit the formation of free radicals from their unstable precursors are called preventive antioxidants, and those which interrupt the radical chain reaction (propagation and branching) are the ‘chain-breaking’ antioxidants (Ou *et al.*, 2001).

DPPH Assay

DPPH method is widely used to test the ability of compounds as free radical scavengers or hydrogen donors and to evaluate the antioxidative activity of plant extracts (Martins *et al.*, 2008). From Fig.2 it is clear that DPPH has maximum absorption at 517 nm and the antioxidant reduces it to the yellow coloured diphenyl-picrylhydrazine. The concentration of antioxidant needed to decrease the initial DPPH concentration by 50 % (IC₅₀) is a parameter widely used to measure the antioxidant activity. A lower IC₅₀ value indicates a higher antioxidant power. The etheric extract has the strongest inhibition (58.7 %) against DPPH radical using 25 µg/mL while methanolic extract gave inhibition value of (51.3 %) at the same concentration.

Figure 3. shows the inhibition percentage of the radical scavenger DPPH for different concentrations (10 – 25 µg/ml) of the EE and ME compared to the standard vitamin C (Vc). Based on the IC₅₀ values, the ability of the extracts and standard to scavenge the DPPH free radical can be given in the following order:

Vc (10.1 µg/mL) > EE (18.17 µg/mL) > ME (23.9 µg/mL).

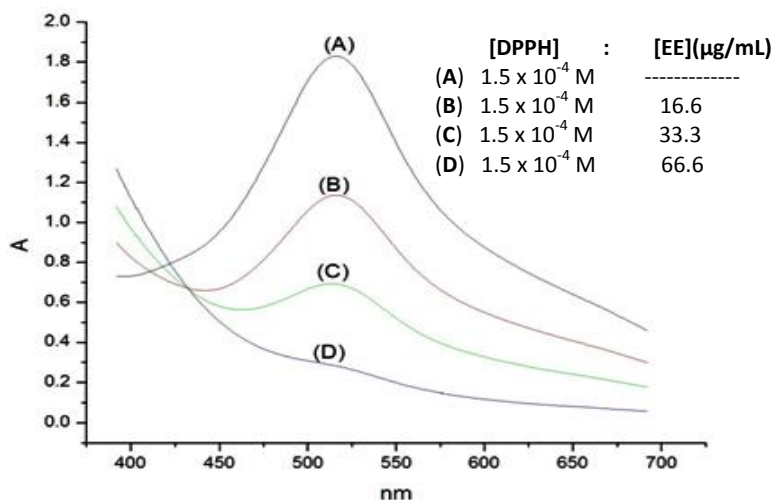


Fig.2. Effect of etheric extract (EE) concentration on the UV-visible spectra of DPPH in DMSO solvent at 25 °C.

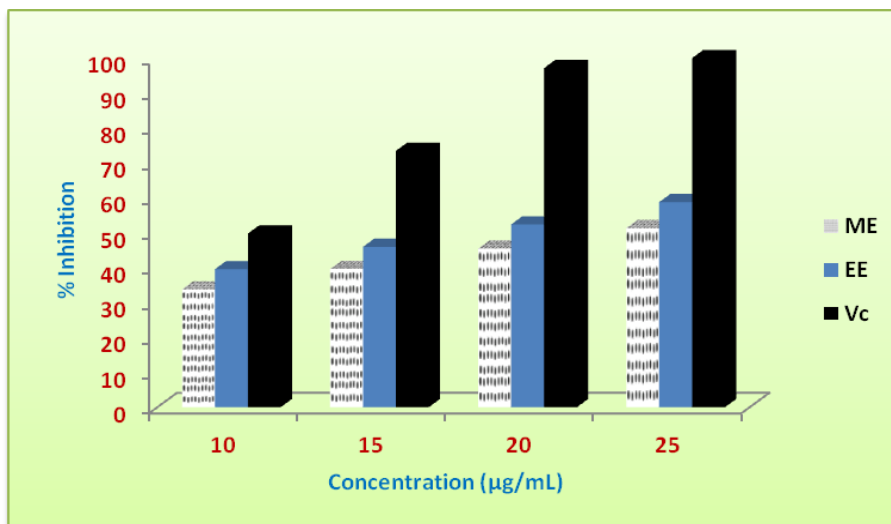


Fig. 3. Effect of different concentrations for EE, ME and Vc on % inhibition of DPPH.

Scavenging of Nitric Oxide Radical

Nitric oxide, generated from sodium nitroprusside in aqueous solution at the physiological pH (7.4), interacts with oxygen to produce nitrite ions, which is measured by Griess reaction (Sangameswaran *et al.*, 2009). In addition to reactive oxygen species, nitric oxide is also implicated in inflammation, cancer and other pathological conditions (Nabavi *et al.*, 2008). In this study, Lawsone and extracts (EE, ME) were added to compete with oxygen leading to produce nitric oxide. Figure 4 summarizes the effect of different concentrations of lawsone, EE, ME extracts and the standard quercetin on the % inhibition of nitric oxide radical scavenger. The % inhibition was increased with increasing concentration of the lawsone and extracts. The etheric extract has shown to be a better reducing power than that of both metanolic extract and the standard (Quercetin). However, activity of lawsone was higher than that of standard and extracts.

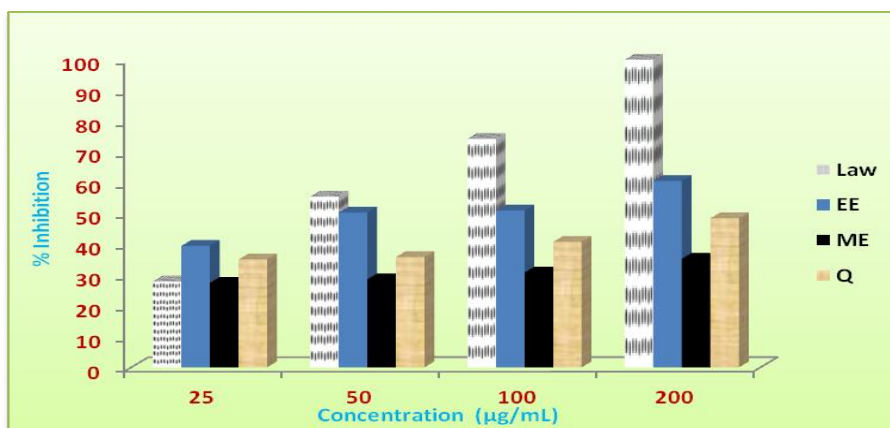


Fig. 4. Nitric oxide scavenging activity of lawsone, ME and EE of *Lawsonia inermis* using quercetin (Q) as reference standard

Superoxide Dismutase Activity Assay

Superoxide anion plays an important role in plant tissues and is involved in the formation of other cell-damaging free radicals (Duan *et al.*, 2007). Superoxide anions are generated *in vitro* by the phenazine methosulphate/NADH system and monitored by the reduction of nitroblue tetrazolium. Figure 5 shows the effects of scavenging superoxide anions by the EE and ME extracts. Maximum superoxide anion inhibition (Figure 5) was shown by EE (72.0 %) and then ME (56.9 %) at the concentration 200 µg/ml. It has been reported that, the increasing order in the superoxide anion scavenging activity was due to presence of hydroxyl group of the phenolics which may contributes to its superoxide anion scavenging activity by their electron donation (Bravo, 1998). Similarly, in this study EE extract might scavenge the superoxide anion in a similar way since they are rich in phenolics containing more hydroxyl groups.

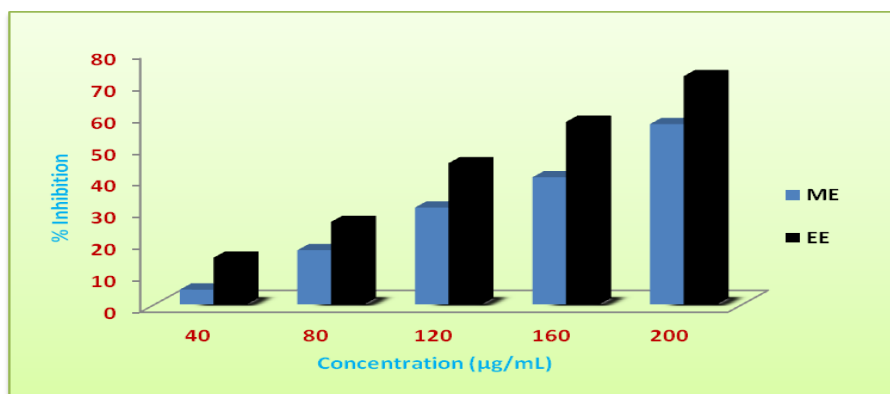


Fig. 5. Superoxide anion scavenging activity of different concentration of ME and EE extracts of *Lawsonia inermis* leaves.

Scavenging of Hydroxyl Radical

The hydroxyl radicals (HO^\cdot) in aqueous media were generated through the Fenton system ($\text{Fe}^{2+}/\text{EDTA}/\text{H}_2\text{O}_2$). The hydroxyl radical bleached the safranin, so decreased the absorbance of the reaction mixture indicating a decrease in hydroxyl radical scavenging ability (Winterbourn and Sutton, 1984).

In the site-specific reactions, methanolic extract at concentrations from 160 to 480 $\mu\text{g}/\text{mL}$, inhibited the production of hydroxyl radicals by 10.0 – 75 % (Fig. 6). Further more, methanolic extract was high in their total polyphenol and total flavonoid contents. Phenols act as antioxidants and scavenge the hydroxyl radical generated from the Fenton reagent. Attempts to use lawsone and etheric extracts as scavengers of hydroxyl radical under the same conditions were unsuccessful.

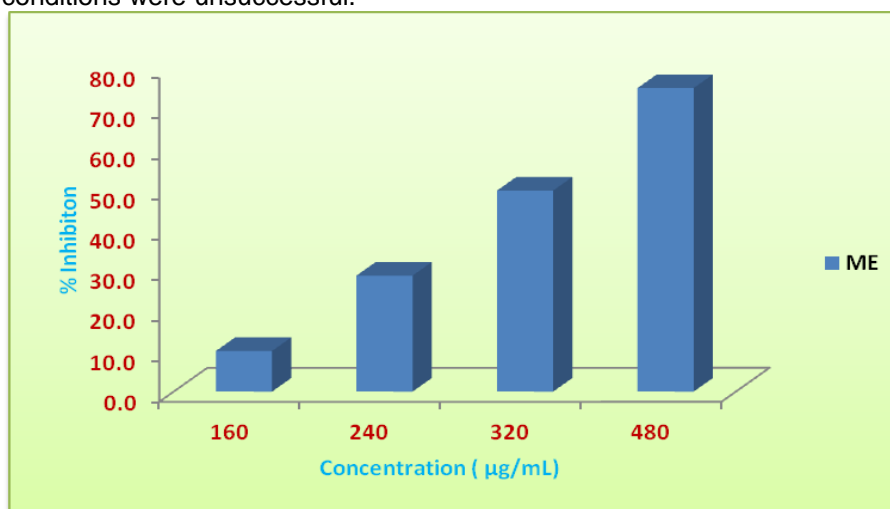


Fig. 6. Hydroxyl radical scavenging activity of different concentration of methanolic extract (ME).

Reducing Power Assay

The more antioxidant compounds convert the oxidation form of iron (Fe^{+3}) in ferric to ferrous chloride (Fe^{+2}). In this assay, the henna extracts were able to convert the oxidized form of Fe^{3+} into the reduced form Fe^{2+} (Fig. 7). The reducing power capacity of the methanol and etheric extracts, with absorbance value of (0.411) and (0.614), respectively were significantly less than that of vitamin C (2.418) at $25 \mu\text{g mL}^{-1}$. The reducing properties are generally associated with the presence of reductones. The antioxidant action of reductones is based on the breaking of the free radical chain by donating a hydrogen atom (Gordon,1990). The result indicated that the marked reducing power of the henna extracts seems to be due to presence of polyphenols which may act in a similar effect as reductones by donating the electrons and reacting with free radicals to convert them into more stable products and terminate free radical chain reaction.

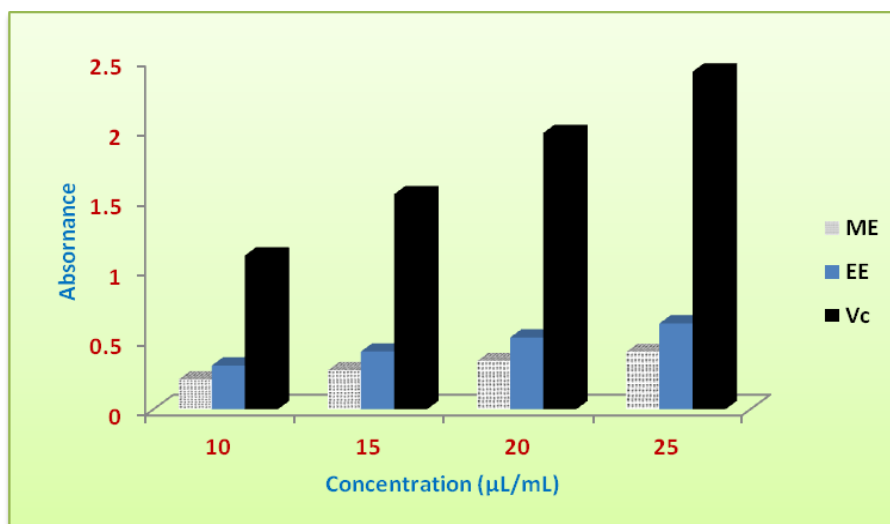


Fig. 7. Reducing power assay of different concentration of ME and EE extracts using ascorbic acid (Vc) as reference standard.

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المواد الكيميائية النباتية والقدرة على مسك الشوارد الحرة لمستخلصات من اوراق الحناء

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في الدراسة الحالية تم استخدام مستخلصين لاوراق نبات الحناء (مستخلص ميثانولي ، مستخلص ايثيري) بالاضافة الى المادة الفعالة لاوراق نبات الحناء (اللوسون) كمضادات اكسدة طبيعية لكبح جماح الشوارد الحرة. وقد قدرت كمية الفلافونات والفينولات الكلية في كلا المستخلصين وتبين ان المستخلص الايثيري يحتوي على تركيز عالي منهما قدرة 67.4 و 150.8 ملجرام/جرام مستخلص على التوالي. ثم اجري تفريد للمركبات الفينولية الموجودة في اوراق نبات الحناء باستخدام الكروماتوغرافيا السائلة عالية الضغط وتبين وجود مركبات فينولية متنوعة بتركيزات مختلفة. كذلك اجريت تجربة لتوضيح القوة الاختزالية للمستخلصين وتبين تفوق المستخلص الايثيري على المستخلص الميثانولي.

اجريت اختبارات معملية لاثبات قدرة المستخلصين واللوسون على مسك انواع مختلفة من الشوارد الحرة مثل DPPH، السوبر اكسيد، النيترواكسيد والهيدرواكسيد. وقد تفاوتت قيم تقدير النشاط المضاد للاكسدة بطريقة تثبيط الشوارد الحرة DPPH حيث اعطى المستخلص الايثيري تثبيط الجذور الحرة بكمية قدرة 18.17 ميكروجرام/مل من المستخلص الايثيري اللازم لأكسدة 50 % من مركب DPPH. وكذلك كان افضل من المستخلص الميثانولي في مسك السوبراكسيد. بينما اعطى اللوسون اعلى مسك للنيترواكسيد يفوق المادة القياسية (الكيروستين). اما المستخلص الميثانولي فله قدرة كبيرة على مسك الشوارد الحر الهيدروكسيدية.

قام بتحكيم البحث

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