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Research Paper

Assessment of antioxidant, anti-inflammatory, anti-cholinesterase and cytotoxic activities of Henna (*Lawsonia inermis*) flowers

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ABSTRACT

Lawsonia inermis flowers were evaluated for chemical and biological properties for the first time. Extracts were prepared successively by hexane, chloroform and methanol. Phenolics (7.8-239.0g gallic acid equivalent/kg of dry mass), tannins (12.5-148.5mg cathechin equivalent/kg of dry mass) and flavonoids (36.2-43.75mg quercetin equivalent/kg of dry mass) were quantified. Methanol extract has a high antioxidant activity (IC_{50}=8.5mg/L by DPPH assay and IC_{50}=5mg/L by ABTS assay) and a good anti-inflammatory activity against 5-Lipoxygenase (IC_{50}=49.33mg/L) compared to references. However, extracts exhibited a poor anti-Alzheimer and anti-diabetic activities. Chloroform extract exhibited a higher anticancer activity (IC_{50}=20mg/L). We observed high correlations between phenolics and flavonoids with antioxidant and anti-inflammatory activities (0.75<R2<0.97). Finally, a very good correlation between anti-inflammatory activity and antioxidant activity with R2=0.99. It may be interpreted that the greatest anti-inflammatory activity was excreted by the best antioxidants extracts, which contain the high amounts of total phenolics.

Key words: *Lawsonia inermis*; Phenolics; Extract; Antioxidant activity; Biological activities.

INTRODUCTION

Tunisian flora is known for its diversity of medicinal plants such as *Lawsonia inermis* (syn. *Lawsonia alba*), commonly known as 'Henna'. *L. inermis* is a sole species in the genus in the family Lythraceae (Arun et al., 2010). Henna is a much branched glabrous shrub or small tree, cultivated for its leaves although stem bark, roots, flowers and seeds have also been used in traditional medicine (Muhammad & Muhammad, 2005). *L. inermis* frequently cultivated in India, the Middle East and along the African coast of the Mediterranean Sea (Hsouna et al., 2011).The yield of *L. inermis* flowers essential oil is in the range 0.01-0.02%, with brown or dark brown color and strong fragrance. The flowers are small and contain ß-ionone as major volatile component (Jacob et al., 2010).

Modern pharmacological research of Henna and its constituents has confirmed its anti-inflammatory, antipyretic and analgesic effect (Prosen et al., 2005). Phytochemical investigations of *L. inermis* leaves have shown predominantly the presence of phenolics (coumarins, flavonoids, naphthalene and gallic acid derivatives) which could be glycosylated (Hsouna et al., 2011). Other compounds, such as triterpenoids, steroids and aliphatic hydrocarbons have been also isolated from leaves of this plant (Siddiqui & Kardar, 2001).

To our knowledge, all studies were concentrated in leaves of *L. inermis*. Few data reported in the literature regarding the chemical composition and biological activity of flowers from Henna. Only Abdelmalek et al. (2014) were studied antioxidant, and anti-acetylcholinesterase activities of aqueous extract of *L. inermis* flowers. Globally, *L. inermis* is interesting natural resource for cosmetic and therapeutic industry. Hence, our choice is based on flowers of Henna which as raw material for extraction of essential oils and various extracts. No data was reported in literature regarding on 5-Lipoxygenase inhibition activity, anti-diabetic and cytotoxic activity of flowers from Henna.

The aim of present study was: (i) to determine the chemical composition (phenolics, tannins, flavonoides) of various extracts (hexane, chloroform and methanol) from *L. inermis* flowers, (ii) screening of their antioxidant, anti-inflammatory, anticancer, anti-Alzheimer and anti-diabetic activities.

MATERIALS AND METHODS

Collection of plant material: *L. inermis* flowers were collected from Gabes (Tunisia, 33.53 N and 10.03 E) in May 2011. The climate in this region is arid inferior with a mild winter. Specimens was identified at the laboratory Arid-Culture and Culture Oasienne, National Institute of Arid Region (IRA, Tunis) and voucher specimen (number: Law 1) was deposited at the Herbarium of the Department of Botany in the cited institute.

Extraction: The fresh flowers were dried in the shade at room temperature and were powdered. 60 g of powder flowers were extracted successively in a Soxhlet system with 300ml of different solvents of decreasing polarity: hexane (10h at 45°C), chloroform (10h at 45°C) and methanol (10h at 65°C). All organic extracts were concentrated by rotary evaporation under vacuum at 35°C. These fractions were kept at 4°C in the dark until further analysis.

Determination of total phenolic compounds by the Folin-Ciocalteu method: The phenolics of extracts were determined method cited by Bekir et al. (2013). The solution of each extract (0.5ml) was mixed with Folin-Ciocalteu reagent (0.2N, 2.5ml). This mixture was allowed to stand at room temperature for 5 min and then Na_2CO_3 solution (75g/L in water, 2ml) was added. After 1h of incubation, absorbance was measured at 765 nm against appropriate solvent. A standard calibration curve was plotted using gallic acid (0-300mg/L). The results were expressed as g of gallic acid equivalent (GAE)/kg of dry mass.

Condensed tannin content: Catechins and proanthocyanidins reactive to vanillin were analyzed by the vanillin method cited by Bekir et al. (2013). One milliliter (1ml) of each extract solution was placed in a test tube and 2ml of vanillin (1% in H_2SO_4 7M) in an ice bath and then incubated at 25°C. After 15min, the absorbance of the solution was read at 500nm. Concentrations were calculated as g catechin equivalents (CE)/kg dry mass from a calibration curve.

Total flavonoids determination: The total flavonoids were estimated according to the method cited by Bekir et al. (2013). A diluted solution (4ml) of each extract was

mixed with a solution (4ml) of aluminium trichloride ($AlCl_3$) in methanol (2%). The absorbance was read at 415nm after 15min. against a blank sample consisting of a methanol (4ml) and extract (4ml) without $AlCl_3$. Quercetin was used as reference compound to produce the standard curve, and the results were expressed as g of quercetin equivalents (QE)/kg of dry mass.

Determination of total anthocyanin content: Total anthocyanin content was measured with the pH differential absorbance method (Bekir et al., 2013). Absorbance of extracts were measured at 510 and 700nm in buffers at pH 1.0 (hydrochloric acid-potassium chloride, 0.2M) and 4.5 (acetic acid-sodium acetate, 1M). The wavelength reading was performed after 15min. of incubation. Anthocyanin content was calculated using a molar extinction coefficient (ε) of 29600 (cyanidin-3-glucoside) and absorbance of $A=[(A_{510}-A_{700})_{pH\ 1.0}-(A_{510}-A_{700})_{pH\ 4.5}]$. Results were expressed as g cyanidin-3-glucoside equivalent (C3GE)/kg of dry mass.

Free radical scavenging activity DPPH test: Antioxidant scavenging activity was studied using 1,1-diphenyl-2-picrylhydrazyl free radical (DPPH) as described by Bekir et al. (2013). 1.5ml of various samples (pure antioxidants or plant extracts) were mixed with 1.5ml of a 0.2mM methanolic DPPH solution. After an incubation period of 30min at 25°C, the absorbance was measured at 520nm ($A_{(sample)}$). A_{blank} experiment was also the same procedure but without sample. The free radical-scavenging activity was then calculated as percent inhibition according to the following equation: $\%\ inhibition=100(A_{(blank)}-A_{(sample)})/A_{(blank)}$

Antioxidant activity extracts was expressed as IC_{50}, defined as the concentration of the test material required to cause a 50% decrease in initial DPPH concentration. Ascorbic acid was used as a standard. All measurements were performed in triplicate.

ABTS radical-scavenging test: The radical scavenging capacity of the samples for the ABTS (2,2'-azinobis-3-ethylbenzothiazoline-6-sulphonate) radical cation was determined as described by Bekir et al. (2013). ABTS was generated by mixing a 7mM of ABTS at pH 7.4 (5mM NaH_2PO_4, 5mM Na_2HPO_4 and 154mM NaCl) with 2.5mM potassium persulfate followed by storage in the dark at room temperature for 16h before use. The mixture was diluted with water to give an absorbance of 0.70±0.02 at 734nm. For each sample, extract (100µL) was allowed to react with fresh ABTS solution (900µL), and then the absorbance was measured 6min after initial mixing. Ascorbic acid was used as a standard. The capacity of free radical scavenging IC_{50} was determined using the same previously used equation for the DPPH method. All measurements were performed in triplicate.

Anti-inflammatory activity: The activity was evaluated like method cited by Bekir et al. (2013). Various concentrations of 20µl of extracts (DMSO) was mixed individually with sodium phosphate buffer (pH 7.4) containing 5-Lipoxygenase (500U) and 60µl of linoleic acid (3.5mM), yielding a final volume of 1ml. However the blank does not contain the substrate, but will be added 30µl of buffer solution. The mixture was incubated at 25°C for 10min, and the absorbance was determined at 234nm. The percentage of enzyme activity was plotted against concentration of the flower extract. The IC_{50} value is the concentration of the flower extract that caused 50% enzyme inhibition.

Cytotoxicity evaluation: Cytotoxicity of sample was estimated on human breast cancer cells (MCF-7) (Belkir et al., 2013). Cell lines were distributed in 96-well plates at $3x10^4$ cells/well in 100µl, and then 100µl of culture medium containing sample at various concentrations were added. Cell growth was estimated by (^3H)-hypoxanthine incorporation after 48h incubation. The (^3H)-hypoxanthine incorporation in the

presence of sample was compared with that of control cultures without sample (positive control being doxorubicin).

Anti-Alzheimer activity: Acethylcholinesterase (AChE) inhibitory activities were measured using Ellman's method, as cited by Bekir et al. (2013). 50µl of 0.1M sodium phosphate buffer (pH 8.0), 25µl of AChE solution, 25µl of extract and 125µl of DTNB were added in a 96-well microplate and incubated for 15min at 25°C. The reaction was then initiated after addition of 25µl of acetylthiocholine iodide. Absorbances were at a wavelength of 412nm. The percentage of inhibition was calculated from (1-S/E)×100, where E and S were the respective enzyme activities without and with the test sample, respectively. Galantamin was used as positive control.

Anti-diabetic activity: α-amylase inhibitory activity was determined using a literature method (Hansawasdi et al., 2000). Starch azure (2mg) was used as substrate and was suspended in 0.2ml of 0.5 M Tris-HCl (pH 6.9) containing 0.01M $CaCl_2$. The tubes containing substrate solution were boiled for 5 min. After 5min at 37°C, 0.2ml of extract (DMSO) was added. 0.1ml of α-amylase in Tris-HCl buffer was added and then incubated at 37°C for 10min. and the reaction was stopped by adding 0.5ml of 50% acetic acid. The reaction mixture was centrifuged at 3000rpm for 5min at 4°C and the absorbance was measured at 595nm. Acarbose was used as positive control. The percentage of inhibition was calculated from (1-S/E)×100, where E and S were the respective enzyme activities without and with the test sample, respectively.

Statistical analysis: All data were expressed as Means±Standard deviations of triplicate measurements. The statistical effects of variety were analyzed by ANOVA test, the least significant difference LSD test was used to assess the significance of differences. The confidence limits were set at $P<0.05$. Correlations were carried out using the EXEL program.

RESULTS AND DISCUSSION

Chemical composition

Extraction yields: The yields of different extracts obtained from flowers of Henna are presented in **Table 1**. Different solvents of increasing polarity were employed successively: hexane, chloroform, and methanol. The highest yields (18.3%) were achieved using the polar solvent methanol. The yield of hexane (2.2%) extract of Henna was significantly 2 times higher than those obtained with chloroform (1.1%). This variation might be due to the polarities of the different compounds in the flowers extracts. No data was reported in the literature regarding the extraction yields of flowers from Henna.

Table-1: Chemical Composition of *Lawsonia inermis* flowers extracts.

Extract	Yields (%)	Phenolics (eq gallic acid) g/kg dry mass	Tannins (eq catechin) mg/kg dry mass	Flavonoids (eq quercetin) mg/kg dry mass
Hexane	2.2	7.8±3.7	64.3±5.7	36.2±4.4
Chloroform	1.1	68.8±2.2	148.5±1.5	43.3±3.2
Methanol	18.3	239±3.3	12.5±4.3	43.8±2.6

Phenolics content: Total phenolics were expressed as g gallic acid equivalents (GAE)/kg of extract (**Table 1**). The amount of total phenolics in the different extracts ranged from 7.8-239g GAE/kg of dry mass. The highest amount of phenolics was obtained by the methanolic extract (239g GAE/kg of dry mass). Polar fraction has more phenolics than non-polar fractions. This variation is due to the better solubility

of phenolic constituents in methanol. We noticed that only Abdelmalek et al. (2014) was quantified the content of phenolics in aqueous extracts of Henna flowers (18.74±1.76g/kg dry mass). This water content is very low compared to the one we obtained with the polar solvent (methanol). No data was reported in the literature regarding the content of phenolics of Henna flowers using organic solvants. Elfalleh et al. (2012) indicates that methanol flowers extracts from *Punica granatum* (species belongs to the same family *Lythraceae* of Henna) showed a lower total phenolics (66.29 ± 3.06g GAE/kg DW), nearly 4 times the total phenolics of our plant.

Tannins content: The amount of tannins in the chloroform flowers extract was the highest (148.5±1.5mg CE/kg of dry mass), followed by the hexane extract (64.3±5.7mg CE/kg of dry mass) and the methanol extract (12.5±4.3mg CE/kg of dry mass). This variation might be due to the better solubility of tannins in chloroform. Tannins represented a little part of total phenolics content in flowers (~0.071%). This is the first study to record the tannins content of flowers extracts of *L. inermis*.

Total flavonoids content: Chloroform and methanol flowers extracts of *L. inermis* displayed similar flavonoids content (43.3-43.8mg QE/kg dry mass). Hexane extract exhibited lower flavonoids content (36.2mg QE/kg dry mass). Flavonoids were in small amounts compared to phenolics. Abdelmalek et al. (2014), obtained 6.29±0.14mg QE/kg dry mass of aqueous extract from Henna flowers. This result showed that the content of flavonoids was six times less important compared to what we found. There are no others studies in the literature that investigated the content of flavonoids in flowers from *Lawsonia inermis*.

No anthocyanins were found in the flowers extracts of *L. inermis.*

Antioxidant activity: This activity was evaluated by the determination of the IC_{50} values, corresponding to the amount of extract required to scavenge 50% of DPPH radical or ABTS cation radical present in the reaction mixture. High IC_{50} values indicate a low antioxidant activity (**Table 2**). The methanolic extract, who exhibited a stronger phenolics content (239g GAE/kg of dry mass), was the most potent radical scavenger when IC_{50}=8.5±3.6mg/L by DPPH assay and IC_{50}=5±1mg/L by ABTS assay. Chloroform and hexane extracts did not show any antioxidant activity (IC_{50}>100mg/L) for both assays. We can report that methanol is a better solvent for more consistent extraction of antioxidant substances from *L. inermis* flowers as compared to other solvents. Comparing the results of ABTS assay to those of the DPPH assay, we can deduce that ABTS assay generally presents more activity (El Babili et al., 2010; Khlifi et al., 2013). The ABTS reactions involve electron transfer and take place at a much faster rate compared to DPPH. This strong activity of the methanolic extract is very interesting, although the extract is a mixture of several compounds, the IC_{50} is comparable with that of the vitamin C (IC_{50}=4.1±1.5mg/L by DPPH assay and 3±1.6mg/L by ABTS assay). This result is encouraging to identify the molecules responsible for this activity.

Our results showed that Henna flowers had a very good antioxidant activity compared to Abdelmalek et al. (2014) with a little antioxidant activity found in aqueous extracts from *L. inermis* flowers by DPPH assay (IC50=0.179±0.02mg/ml). The difference between our results and those results can be attributed to the different methods of extraction and solvents extraction. We have used the Soxhlet, while they were used a simple aqueous maceration, harvesting time, and growing conditions.

Table-2: Antioxidant and anticancer activities of *Lawsonia inermis* flowers extracts.

Extract	DPPH assay (IC_{50} mg/L)	ABTS assay (IC_{50} mg/L)	Anticancer activity (IC_{50} mg/L)
Hexane	>100	>100	>100
Chloroform	>100	>100	21±3
Methanol	8.5±3.6	5±1	50±4
Vitamin C	4.1±1.5	3.0±1.6	
Doxorubicin			0.2±0.0

Anti-inflammatory activity: In the present study, chloroform and n-hexane extracts showed a low anti-inflammatory activity with 31.85% and 26.25%, respectively at 200mg/L (**Table 3**). Methanol extract showed the best inhibition percentage (87.7%) at 200 mg/L. This strong activity of the methanolic extract is very interesting with IC50=49.3±2.3mg/L.

The high total phenolics content found in methanol extract imply the role of phenolics in contributing to this activity (Oueslati et al., 2012). According to the literature the greatest anti-inflammatory activity was exerted by the best antioxidants extracts, which contain the high amounts of total phenolics. These results obtained indicated that methanol extract of *L. inermis* can be a potential source of anti-inflammatory and antioxidants agents.

This is the first study to evaluate the anti-5-Lipoxygenase activity of flowers from Henna.

Table-3: Anti-diabetic, Anti-Alzheimer and anti-inflammatory activities of *Lawsonia inermis* flowers extracts.

Extract	Anti-inflammatory activity (% at 200mg/L)	Anti-diabetic activity (% at 200mg/L)	Anti-Alzheimer activity (% at 200mg/L)
Hexane	26.3±3.1	nd	nd
Chloroform	31.9±1.3	10.1±0.7	29.7±1.1
Methanol	87.0±9.5 (IC_{50} 49.3±2.3 mg/L)	53.6±4.2	38.1±2.4
NDGA (2.5mg/L)	96.1±4.7		
Acarbose (50mg/L)		82.5±4.3	
Galantamin (0.7mg/L)			73.0±6.5

- NDGA: Nordihydroguaiaretic acid. nd: not detected.

Anti-diabetic activity: The percentage of inhibition against α-amylase of all our extract not exceeds 50% at 200mg/L. We can deduce that phenolic compounds lonely can't be the chief actors for all activities, but there are other compounds that can be responsible. *P. granatum* flowers extracts possesses evident anti-Alzheimer and anti-diabetic activities (Arun et al., 2012). It is important to note that the flowers of this species of *L. inermis* have not been evaluated anti-diabetic capacity.

Anti-Alzheimer activity: In this study a lower inhibition percentage (<50% at 200mg/L) against the enzyme acetylcholinesterase was observed for the Chloroform (29.67%) and methanol (38.05%) flowers extracts of Henna (**Table 3**). Our result was in good agreement with Abdelmalek et al. (2014), who indicated that flowers extracts of Henna were found to be inactive against acetylcholinesterase enzyme. This study showed that only leaf extracts of *Lawsonia inermis* possessed the best capacity for inhibiting acetylcholinesterase enzyme.

Anticancer activity: To determine the antitumor activity of all extracts against HCT-116 cells, cytotoxicity MTT assay was tested by *L. inermis* extracts. The chloroform

fraction, who exhibited a higher tannins content (148.5±1.5mg CE/kg of dry mass), has the stronger anticancer activity with IC_{50}=21mg/L. The methanol extracts was weaker with less growth inhibition of HCT-116 cells with IC_{50}=50mg/L. We can deduce that chloroform is a better solvent for more extraction of anticancer compounds from *L. inermis* as compared to other solvents.

Yusuf et al. (2012) reported that *L. inermis* has several pharmacological uses such as antitumor, anthelmintic and burn wound healing. Arun et al. (2010) said that lawsone (2-hydroxy-1,4-naphthoquinone) is an active naphthoquinone derivative isolated from Henna leaves which gives the cytotoxic properties. Another work indicated that lawsone is chief coloring component of Henna and it is not mutagenic, but toxic to the cells in dose-dependent manner (Surveswaran et al., 2007). Akrout et al. (2011) said that there is a good correlation between the inhibition of cancer cells proliferation and the levels of phenolics and flavonoids contents.

There are no other studies in the literature that investigated human breast cancer cells (HCT116) against *L. inermis* flowers extracts.

Correlation: We observed that the content of phenolics and flavonoids in flowers extracts from Henna was correlates with their antioxidant and anti-inflammatory activities. The R^2 correlations coefficients between the DPPH assay (or ABTS assay) and phenolics and flavonoids were 0.93 and 0.75, respectively. We have found a correlation between the inhibition of 5-Lipoxygenase and the contents of phenolics and flavonoids (R^2=0.97 and 0.82, respectively). Finally, a very good correlation between anti-inflammatory activity and antioxidant activity (DPPH assay) with R2=0.99. It may be interpreted that the greatest anti-inflammatory activity was excreted by the best antioxidants extracts, which contain the high amounts of total phenolics.

CONCLUSION

Thus methanolic extract has the highest *in vitro* antioxidant activity and could be considered as natural source of strong antioxidant substances in food industries.

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