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In vitro Antioxidant and anti-inflammatory properties of selected Moroccan medicinal plants

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ABSTRACT

The aim of the present study was to examine the antioxidant and anti-inflammatory activities of three Moroccan ethno medicinally important plants. The antioxidant activity was performed using β-Carotene bleaching assay, DPPH, ABTS and Ferric reducing assays. *In vitro* anti-inflammatory assays was also studied through the evaluation of membrane stabilization potential and inhibition of protein denaturation. Results revealed that *Lawsonia inermis L* leaves extract had significantly higher phenolics-content (5.23 g GAE/100g DW) and exhibited the highest antioxidant activity based on DPPH (IC_{50} = 18.26 µg/mL). The highest flavonoids content was found in *Rosa damascena*. L flower (3.97 RE/100g DW) which shows the highest antioxidant activity based on ABTS (30.39 mmol TE/100g DW), FRAP (38.54 mmol TE/100gDW) and β-Carotene bleaching assay (IC_{50} = 56.33 µg/mL). Concerning the anti-inflammatory potency, the highest membrane stabilization effect was found in *Rosa damascena*. L flower (IC_{50} = 125.02 µg/mL) and the highest anti-denaturation of protein effect was found in *Lawsonia inermis L* leaves extract (IC_{50} = 103.21 µg/mL). Consequently, to conclude flavonoids and related phenolics present in studied plant materials may possibly be responsible for the important anti-inflammatory and antioxidant activities.

INTRODUCTION

Inflammation is the normal response of body's immune system that occurs in vascular tissues against harmful stimuli such as pathogens, cellular damage and irritants (Ferrero-Miliani *et al.*, 2007). Chronic inflammation is usually associated with an increase in reactive nitrogen and oxygen species production bringing about an oxidative stress initiated by an imbalance between reactive oxygen species and the biological system's defence able to eliminate these free radicals (Salman and Ashraf, 2013). This oxidative stress has been involved in several diseases including cancer, neurodegenerative diseases, atherosclerosis, malaria, chronic fatigue syndrome and rheumatoid arthritis (Chaitanya *et al*., 2010). Recently there has been an extensive interest in the therapeutic potential of medicinal plants as antioxidants in scavenging such free radical-induced tissue injury (Pourmorad *et al.*, 2006). In southeast Morocco traditional medical system, plants such as *Lawsonia inermis* L leaves, *Rosa damascene* flowers, *Cuminum cyminum* seed are used to treat gastric pains, menstrual disorder, fever, oedema, rheumatism and bronchitis. Several researchers have reported the different biological actions of *Lawsonia inermis* L leaves, *Rosa damascena* flowers, *Cuminum cyminum* seed using various in-vitro and in-vivo test models.

Lawsonia inermis L leaves have been found to exhibit an antioxidant, antimicrobial, antifungal, antidiabetic, anthelminthic, antitrypanosomal, abortifacient, and anticancer activities as well as immunomodulatory, hepatoprotective and wound healing effects (Makhija *et al*., 2011). *Cuminum cyminum* seeds have been revealed to have an antioxidant, antimicrobial, anticarcinogenic, antidiabetic, estrogenic, anti-osteoporotic, antitussive, anti-epileptic, analgesic, antiaggregatory and Immunomodulatory proprieties (Johri. 2011). The experimental studies conducted on *Rosa damascena* have been shown to have an antioxidant, antibacterial, antitussive, antidiabetic, anti-HIV, analgesic, anticonvulsant, anti-anxiety, anti-depressant, hypnotic effects (Boskabady *et al*., 2011).

Based on the traditional claims surrounding *Rosa damascena*, *Lawsonia inermis* L and *Cuminum cyminum*, The aim of this research was to examine the antioxidant and antiinflammatory activities through several in vitro methods.

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MATERIALS AND METHODS

Plant material

The plant materials were harvested during their optimal season; flowers of *Rosa damascena* were collected in May 2013, *Lawsonia inermis* L leaves in June 2013 and the seeds of *Cuminum cyminum* were collected during May 2013.

To insure that there are no impurities on the surface of plant materials, they were washed under running tap water then were air dried under shade using ventilator to dry more quickly. The dried plants were ground to powder using an electrical grinder.

Preparation of rich polyphenol extracts

Five grams of each powdered sample were kept in a glass flask and the volume was made up to 100 mL with the methanol–water (4:1, v/v), after content dissolving. Ultrasonic extraction was performed in a sonication water bath (Branson Ultrasonic Cleaner 2510R) with a working frequency and temperature fixed at 40 KHz and 69°C respectively for one hour.

At the end of sonication, the suspension was cooled to ambient temperature and then filtered using Whatman no. 4 filter paper. The collected filtrate was then used for the determination of total phenolics compounds and to evaluate the antioxidants and anti-inflammatory activity.

Measurement of total polyphenolic content.

The total phenolic contents in plant material were measured according to the method described by (Bouhlali *et al.*, 2015a). Five hundred µL of Folin–Ciocalteau reagent (10-fold diluted with water) was mixed with 100 µL of diluted filtrate, and then 400µL of aqueous sodium carbonate solution (7.5% w/v) was added.

The mixture was allowed for 60 min at room temperature then the absorbance was measured at 765 nm. The calibration curve was prepared using Gallic acid. The total phenolic compounds were expressed as Gallic acid equivalent in g/100 g dry weight (DW) of plant material.

Measurement of flavonoid content:

The total flavonoid content of plant material was determined by the method of (Kim *et al.*, 2003). Five hundred µL of diluted filtrate was mixed with 2 mL of distilled water. Then 150 µL of aqueous sodium nitrite solution (5% w/v) was added, followed by 150 µL of aqueous aluminum chloride solution (10% w/v). Test tubes were incubated for 5 min at room temperature, and then 1 mL of sodium hydroxide (1M) was added to the mixture and then the final volume was made up to 5 mL with distilled water. The mixture was vortexed and the absorbance was determined at 510 nm.

The absorbance was calibrated to a standard curve of Rutin solution and the results were expressed as g Rutin equivalents (RE)/100 g of dry weight (DW) of plant material.

ABTS radical scavenging assay

The ABTS assay was done using the method of (Re *et al.*, 1999). The ABTS radical cations (ABTS+) were produced by reacting aqueous solution of ABTS (7mM) with an aqueous solution of potassium persulphate (2.45mM). The mixture was left at the ambient temperature in the dark for 12-16 hours before use; the result solution was diluted with distilled water to obtain 0.700 ± 0.005 at 734 nm in the absorbance. 30µL of the sample added to 3 mL of the ABTS solution was allowed at ambient temperature. After 6 min the absorbance at 734 nm was recorded immediately. Trolox solution was used to prepare the standard curve and the total antioxidants were expressed as mmol of Trolox equivalents per 100 g of dry weight (DW) of plant part.

DPPH radical scavenging activity

Radical scavenging activity of plant material against stable DPPH was evaluated using the method described by (Bouhlali *et al.*, 2015b) with slight modifications. The reaction mixture contained 100 µL of filtrate at different concentration and 1.9 mL of methanolic DPPH (0.3mM). The result mixtures were left at room temperature for 20 min and the absorbance was measured at 517 nm. The IC_{50} (concentration providing 50% inhibition) values were calculated from the plotted graph of scavenging activity against the concentrations of the samples.

$$\%\ inhibition\ = \frac{\left(\text{Abs (control)} - \text{Abs (sample)}\right)}{\text{Abs (control)}} X100$$

Abs control is the absorbance without extract; Abs sample is the absorbance of the extract or standard.

Ferric reducing antioxidant power assay

The ferric reducing activity of the plant material extract was estimated based on the method of (Benzie and Strain, 1999). The FRAP reagent was prepared by mixing 50 mL of acetate buffer (0.3M, pH 3.6), 5 mL of Ferric chloride solution ($FeCl_3$) (20mM) and 5 mL of TPTZ (2,4,6-Tripyridyl-s-Triazine) solution (10mM) prepared in HCl (40 mM). 2 mL of the freshly prepared FRAP reagent was added to the 10µL of extract. Then the absorbance was measured at 593 nm against the blank after 10 minutes at ambient temperature. Trolox was used to prepare the standard curve. The result was expressed as Trolox equivalent in mmol/100 g of dry weight (DW) plant parts.

β-Carotene bleaching assay

The β-carotene bleaching inhibition method was carried out using the method of (Shahidi *et al.*, 2001). Two mg of β-carotene were dissolved in ten mL of chloroform then four mL of this solution were pipetted into a round-bottom flask, which contains forty mg of linoleic acid and five hundred mg of Tween 40. The chloroform was then, evaporated under vacuum at 40 °C and one hundred mL of oxygenated water was added and vigorously shaken to yield fresh emulsion one mL of the emulsion was transferred into test tubes containing one hundred µL of diluted filtrate and incubated in a water bath at 50 °C then the

absorbance was measured at 470 nm immediately (t = 0 min) and after 120 min of incubation against a blank which contains the emulsion without β-carotene. The antioxidant activity of plants was compared to the positive control, which was BHT in this assay. The β-carotene bleaching inhibition (%) of the analyzed solution was calculated via the following formula:

$$\beta \text{ carotene bleaching inhibition } (\%) = \frac{\beta \text{ carotene content after 2 h assay}}{\text{Initial } \beta \text{ carotene content}} \text{ x } 100$$

The IC_{50} was calculated using a standard curve between the plant material concentration and the percentage of β-carotene bleaching inhibition.

Inhibition of albumin denaturation

The Inhibition of albumin denaturation was assessed according to the modified method of (Chandra *et al.*, 2012). Briefly, one mL of 1% bovine serum albumin prepared in PBS (phosphate-buffered saline, pH 6.4) was added to one mL of varying concentrations of plant filtrate. This mixture was left at ambient temperature for 20 min and then heated at 70°C for 5 min. The resulting mixture was cooled down to the ambient temperature and their turbidities were read at 660 nm. The same procedure was repeated using double-distilled water and the Diclofenac as control and standard respectively. The Percentage inhibition (IP%) of protein denaturation was calculated as follows:

$$\text{Percentage inhibition (IP\%)} = \frac{(\text{Abs Control} - \text{Abs Sample})}{\text{Abs control}} X100$$

Abs control is the absorbance without plant material; Abs sample is the absorbance of the plant material or standard.

The membrane stabilization potential

The membrane stabilization potential was determined according to the method described by (Murugan and Parimelazhagan, 2014). The sterilized Alsever's solution prepared by dissolving 0.05% citric acid, 0.42% of sodium chloride, 0.8% sodium citrate, and 2% dextrose in distilled water was mixed with equal volume blood collected from healthy human volunteers who did not use any NSAIDS for fifteen day's before blood collecting. After centrifugation at 3000 rpm of result blood solution the cell pellet was washed with isotonic Saline (9 g/L) which was used then to prepare a suspension containing 10% cell pellet.

The reaction mixture contains 1 mL of phosphate buffer, 0.5 mL of Diclofenac of plant extract at various concentrations (50, 100, 200, 400 and 800 µg/mL), 0.5 mL of blood suspension (10%) and 2 ml of hypotonic saline (3.6 g/L). After incubation for 30 min at 37°C, the mixtures were centrifuged at 3000 rpm and the hemoglobin content of supernatant was estimated by a spectrophotometer at 560nm.

Statistical analysis

The results were statistically evaluated by one way analysis of variance (ANOVA) using StatView 5.0 software. The experimental results were reported as mean ± SE (standard error) (n=6). Pearson's correlation coefficient (R^2) was used to measure the association between two variables. Differences at $p < 0.05$ were considered significant.

RESULTS AND DISCUSSION

Total phenolic and flavonoid content

The results of total polyphenolic and flavonoid contents from studied plant material are depicted in **Table 1**. The highest phenolic content was found in *Lawsonia inermis L* leaves extract (5.31 g GAE/100 g DW) which is followed by *Rosa damascena. L* flower (4.89 g GAE/100g DW) and the lowest was found in *Cuminum cyminum* seeds extract (1.07 g GAE/100g DW). Regarding total flavonoid contents the highest level was found in *Rosa damascena. L* flower (3.97 RE/100g DW) which was higher than those of *Lawsonia inermis L*. leaves extract (3.62 g RE/100g DW) and *Cuminum cyminum* seeds extract (0.52 g RE/100g DW). The result reported by (Tan *et al.*, 2013) for *Lawsonia inermis* leaves (5.55 g GAE/100 g DW), by (Rebey *et al.*, 2012) for *Cuminum cyminum* seeds (1.45-1.86 g GAE/100 g DW) and by (Bayram *et al.*, 2015) for *Rosa damascena* flower (4.31 g GAE/100 g DW) are very close to our results. Several studies link the antioxidant activity of medicinal plant to their phenolics content due to their redox properties, which plays an important role in neutralizing and scavenging free radicals, quenching singlet and triplet oxygen, or decomposing peroxides (Ghasemzadeh *et al.*, 2010).

Table 1: Antioxidant activity, total phenolic content and total flavonoid of studied plants materials

	Total Phenolic content g GA/100g DW	Total Flavonoid content g RE / 100g DW	FRAP mmol TE/100g DW	ABTS mmol TE/100g DW
Lawsonia inermis L	5.31 ± 0.16	3.62 ± 0.15	33.06 ± 0.19	26.84 ± 1.19
Rosa damascena	4.89 ± 0.13	3.97 ± 0.11	38.54 ± 0.32	30.39 ± 0.84
Cuminum cyminum	1.07 ± 0.07	0.52 ± 0.04	6.89 ± 0.51	2.79 ± 0.43

Values are mean ± SE (standard error). Results are statistically significant at $p < 0.05$. GAE – Gallic acid equivalents. RE – Rutin equivalents. TE- Trolox equivalents

Antioxidant activities

Because the plant extract contains a wide range of antioxidant compounds, which may act through different mechanisms and because there is no single method can evaluate the total antioxidant capacity of these medicinal plants. Hence, the antioxidant activity of selected plant materials was carried out using FRAP, ABTS and DPPH scavenging as well as β-carotene bleaching assay.

The ferric reducing antioxidant power (FRAP) is quick, simple, reproducible, linearly related to the molar concentration of the antioxidants and commonly used to study the antioxidant capacity of plant materials. This assay based on the ability of

antioxidant to reduce ferric (III) iron to ferrous (II) iron through an electron transfer reaction (Shi *et al.*, 2010). Table 1 showed that *Rosa damascena* extract (38.54 mM TE/100g plant material) was the most powerful to reduce the ferric ions into ferrous ions among studied plants, followed by *Lawsonia inermis L* extract (33.06 mM TE/100g plant material) and *Cuminum cyminum* extract *(*6.89 mM TE/100g plant material). This reducing properties are associated with the presence of polyphenol and Flavonoids which may act as reductones as showed the significant linear correlation which is (R^2 > 0.930) between FRAP in one hand and phenolic and flavonoid content in the other hand (Table 3).

DPPH and ABTS methods are simple, precise, inexpensive and commonly used to assess the antioxidant activity of medicinal plant. The DPPH (2,2- diphenyl-1-picrylhydrazyl) and the ABTS (2,2′-azinobis(3-ethylbenzothiazoline 6-sulfonic acid)) in its radical form has a characteristic absorbance at 517nm and 734nm respectively, which vanishes after its reduction by electron or hydrogen donors. This reduction can be monitored by measuring the decrease in its absorbance at 517nm and 734nm (Shalaby and Shanab., 2013). As showed in Table 2 *Rosa damascena* extract was the most effective radical scavenger against both of DPPH (IC_{50} = 27.48 µg of plant materiel/mL) and ABTS (30.39 mM TE/100 g plant materiel). The lowest scavenging ability was observed in *Cuminum cyminum* extract with (2.79 mM TE/100 g plant materiel) for ABTS assay and (IC_{50} = 172.05 µg of plant materiel/mL) for DPPH assay. Our result for *Lawsonia inermis L* using DPPH assay agrees to those reported by (Guha *et al.*, 2011). The result of FRAP and DPPH conducted on *Rosa damascena* is very high than those reported by (Kalcheva-Karadzhova *et al.*, 2014). The results reported by Rebey *et al.*, 2012 using DPPH and β-carotene bleaching methods for *Cuminum cyminum* seeds were very high than the results found in our study.

Concerning the β-carotene bleaching assay based on the reduction of the orange color intensity of β-carotene due to its reaction with peroxyl radical (LOO •) generated by linoleic acid in the presence of oxygen during incubation at 50°C (Moon and Shibamoto, 2009). The difference between the initial absorbance measured at the beginning and the absorbance after 120 min give information about the ability of plant material to inhibit the bleaching of the β-carotene solution. The plant antioxidants can disturb the degree of β-carotene bleaching through neutralization of linoleate-free radicals and other radicals formed in the system (Jayaprakasha *et al.*, 2001). The results have shown that plant extract inhibited the β- carotene bleaching in a dose dependent manner and the strength of inhibition varied between (56.33µg/mL) for *Rosa damascena* extract which presented the lowest IC_{50} and *Cuminum cyminum* extract with the highest IC_{50} *(*279.54 µg/mL) (Table 2). It was speculated that *Rosa damascena* extract *and Lawsonia inermis* extract exhibited an interesting antioxidant activity, which is very close to a standard antioxidant (BHT) which possessed an IC_{50} = 53.25 µg/mL.

As shown in Table 3, there is a strong correlation between total polyphenol contents and the antioxidant activity as well as between total flavonoid contents and the antioxidant activity of samples (R^2 > 0.93). The strong correlation in this study confirms other studies which have exposed that antioxidant activities are associated with plants phenolic content which may act as singlet oxygen quenchers, reducing agents and hydrogen donors (Chang *et al.*, 2001).

Table 2: Antioxidant activity based on DPPH radical scavenging activity and β-Carotene bleaching assay

	IC_{50} of DPPH µg/mL	**β-carotene IC_{50} µg/mL**
Lawsonia inermis L	18.26 ± 1.04	71.32 ± 5.63
Rosa damascena	27.48 ± 2.72	56.33 ± 4.37
Cuminum cyminum	172.05 ± 8.19	279.54 ± 11.67
BHT	22.67± 2.17	53.25 ± 3.62

Values are mean ± SE (standard error). Results are statistically significant at $p < 0.05$.
BHT: Butylated hydroxytoluene

A high correlation was observed between ABTS, FRAP, DPPH and β-carotene bleaching methods (Table 3), suggesting a possible antioxidant activity relationship of the compounds that react in these methods although they have different reaction mechanisms. The strong correlation between FRAP and ABTS assays may be due to the same mechanism that they have and their similar redox potential (0.70 V for ferric reduction and 0.68 V for reaction with ABTS) (Müller et al, 2011).

Table 3: Correlation phenolic and flavonoid content with antioxidant activities.

	Phenolic	**flavonoid**	**FRAP**	**DPPH**	**ABTS**	**β-carotene**
Phenolic	1					
flavonoid	0.967	1				
FRAP	0.937	0.995	1			
DPPH	0.998	0.979	0.953	1		
ABST	0.957	0.999	0.998	0.971	1	
β-carotene	0.978	0.998	0.989	0.987	0.996	1

The anti-inflammation activity

The ethical challenges and the nonexistence of rationale to use animals for pharmacological research of new chemical compounds, when other suitable methods are available or could be investigated, pushed us to select the protein denaturation bioassay and membrane stabilization potential for in vitro evaluation of anti- inflammatory property activity of studied plant materials.

The inhibition of protein denaturation

Denaturation of tissue proteins is well-documented as one of the causes of inflammatory and arthritic diseases, through auto antigen production (Williams *et al.*, 2008) and the ability of plant material to bring down the protein denaturation could be effective against inflammation diseases.

Maximum inhibition of heat induced albumin denaturation was observed from *Lawsonia inermis L* leaves extract (IC_{50}= 103.21 µg/mL) followed by *Rosa damascena* flowers extract (IC_{50}=129.04 µg/mL) and *Cuminum cyminum* seeds extract

(IC_{50}=234.87 µg/mL). From the IC_{50} values, it becomes evident that *Rosa damascena* flowers extract and *Lawsonia inermis L* leaves extract were very effective and close to Diclofenac (IC_{50}= 86.75 µg/mL) which is the standard anti-inflammation drug. The present findings showed that the plant material exhibited a concentration dependent inhibition of thermal protein denaturation. Mizushima and Kobayashi, (1968) using inflammatory drugs (salicylic acid, phenylbutazone etc.) found this inhibition dose dependent. The in vitro anti-inflammatory effect of plant material may be due to synergistic effect rather than single constituent of their polyphenols contents.

The reported results are in agreement with those reported by (Hajhashemi *et al.*, 2010), (Alia *et al.*, 1995) and (Bhat *et al.*, 2014) who has found an important anti-inflammatory effect using Carrageenan-induced rat paw oedema assay by for *Rosa damascena* flowers, *Lawsonia inermis L* leaves and *Cuminum cyminum L* seeds respectively.

The membrane-stabilizing potential

In the course of inflammatory response, the activated neutrophil releases their lysosomal constituents such as proteases and bactericidal enzymes, which cause supplementary tissue inflammation and damage (Murugesh et al, 1981). Because human erythrocytes membranes are analogous to lysosomal membrane components (Mounnissamy *et al.*, 2008), the inhibition of hypotonicity-induced hemolysis was used to investigate the anti-inflammatory activity of plant material. Results presented in **Fig.1** showed that *Rosa damascena* flowers extract exhibited the highest inhibition of hemolysis (IC_{50} = 125.02 µg/mL) followed by *Lawsonia inermis L* leaves extract (IC_{50}= 170.24 µg/mL) and *Cuminum cyminum* seeds extract (IC_{50}= 269.28 µg/mL). The results of erythrocytes protective effect found using these plant extract are lower to those of Diclofenac (IC50= 94.77µg/mL). The membrane-stabilizing effect was established to increase with the increase drug and plant extract concentration. These plant extracts may act through a deformation of cells via their interaction with membrane proteins (Shinde *et al.*, 1999) or other compound in the erythrocyte membranes, which provoke later alteration of the surface charges of the cells (Oyedapo *et al.*, 2004). Knowing that changing in the form and volume of the cell is much related to the intracellular amount of calcium. The membrane protective effect of plant extract may be due to their ability to adjust the intracellular concentration of calcium into the erythrocytes (Chopade *et al.*, 2012). Tannins and saponins are able to bind cations and other biomolecules (Oyedapo *et al.*, 2001) hence their profound stabilizing effect.

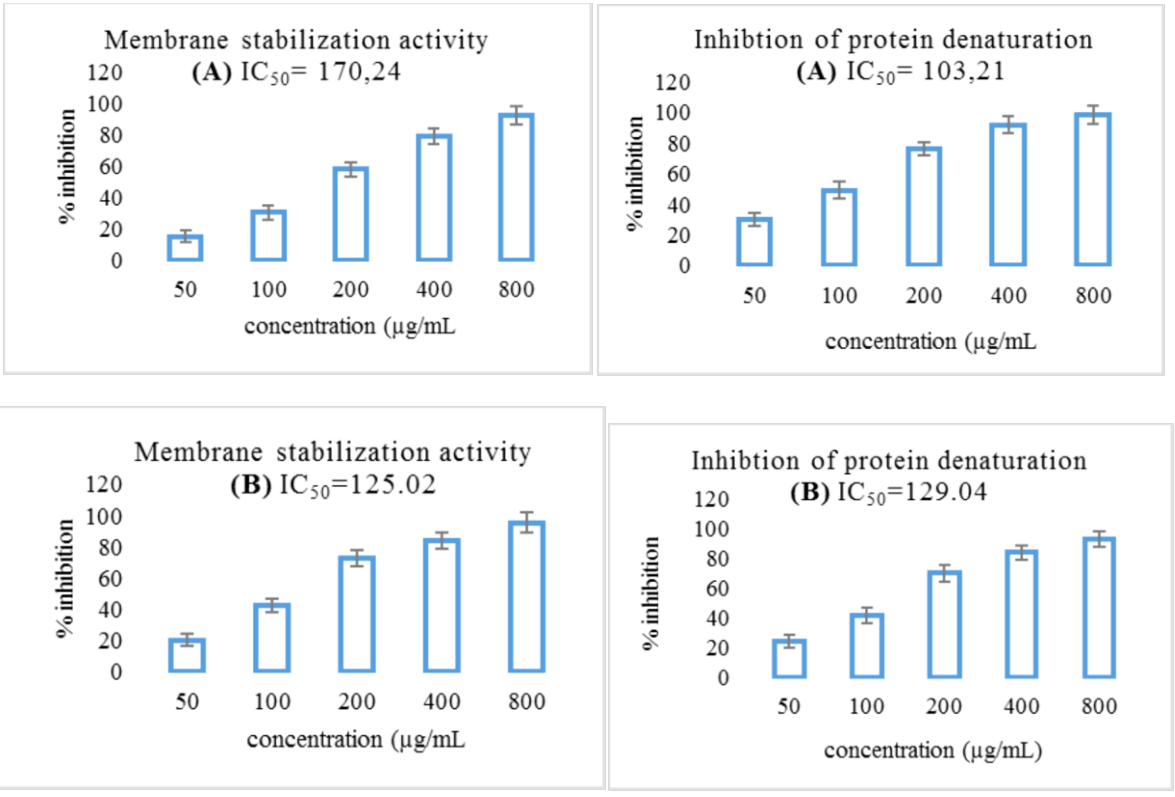

Fig. 1:..

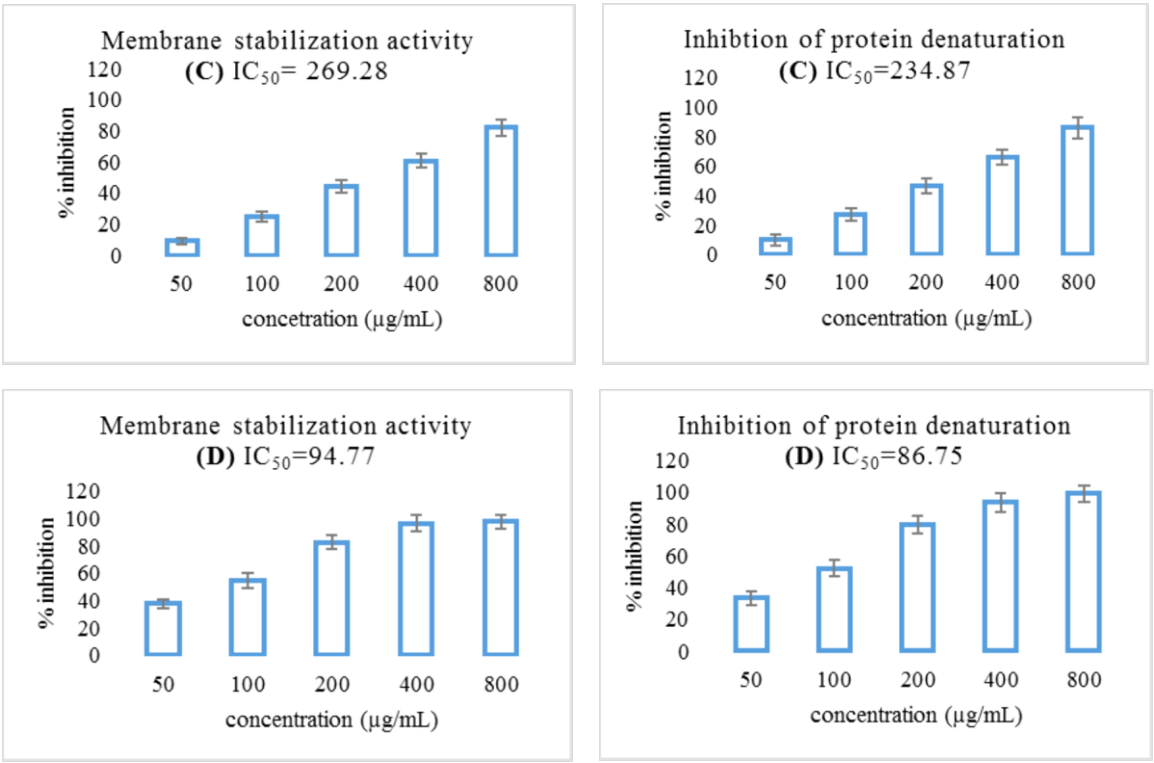

Fig. 1: Anti-inflammatory activity based on Membrane stabilization activity and Inhibition of protein denaturation.
A: *Lawsonia inermis L*, B: *Rosa damascena*, C: *Cuminum cyminum*, D : Diclofenac.
Values are mean ± SE (standard error). Results are statistically significant at $p < 0.05$.

CONCLUSION

Our investigation clearly demonstrates that studied plant materials possess significant antioxidant activity against various antioxidant systems and potent anti inflammatory. The presence of various Polyphenols especially flavonoid may be responsible of these activities. The present study provides a scientific base for the ethno medicinal claims of plants. Further studies are recommended to isolate the active principle responsible for these activities.

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