IN-VITRO ANTI-INFLAMMATORY EVALUATION OF ETHANOL EXTRACTS OF *Moringa oleifera, Thymus vulgaris* AND THEIR 1:1 EXTRACT BLEND ON PROTEIN DENATURATION

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Abstract: *Moringa oleifera and Thymus vulgaris* are well known and widely used herbs, which contains several interesting bioactive constituents and possesses health promoting properties. The aim of the present study is to evaluate and compare the*in vitro* anti-inflammatory effects of ethanol extracts of *Moringa oleifera* and *Thymus vulgaris* and a 1:1 blend of the two extracts against the denaturation of proteins. The test extracts and the reference drug of varying concentrations were incubated with egg albumin under controlled experimental conditions and subjected to determination of absorbance to assess the anti-inflammatory property. A standard anti-inflammatory drug, Ibuprofen, was used as reference drug. The results obtained showed a concentration-dependent inhibition of protein (albumin) denaturation by both extracts, including the 1:1 blend of the extractsas well as the reference drug. The extract concentrationas well as the reference drugfor 50% inhibition (EC_{50}) was determined by the dose-response curve using GraphPad Prism 6.01 software package and were as follows: *Moringa oleifera* (EC_{50} = 215.9±29.8 µg/mL); *Thymus vulgaris* (EC_{50} = 144.9±1.1 µg/mL)*;* the extracts blend (EC_{50} = 441.6±8.6 µg/mL) and ibuprofen (EC_{50} = 1599±337 µg/mL), respectively. These findings showed that both *Moringa oleifera* and *Thymus vulgaris* possessed marked *in vitro* anti-inflammatory effect against the denaturation of protein when compared with the standard anti-inflammatory drug, ibuprofen. *Thymus vulgaris* extractwas found to be more active than *Moringa oleifera*, possibly due to the higher flavonoids and/or organic acid contents.

Keywords: *Moringa oleifera*, *Thymus vulgaris*, Ibuprofen, anti-inflammation, protein denaturation

Introduction

Herbs and vegetables are important components of human diet and give protections against many common illnesses and diseases, especially cardiovascular diseases, high blood pressure, stroke, oxidative stresses, cancer, obesity, diabetes and neurodegenerative conditions (Kavalcová *et al.*, 2014). Various spices and herbal extracts are used for preservation of food, as well as appetizers; and the medicinal value of plants lies in the presence of different phytochemical components (tannins, alkaloids, terpenoids, flavonoids and phenolic compounds) that bring particular physiological effect in human body (Mousavi *et al.*, 2011; Javed *et al.*, 2013). The world health organization estimated that 80% of the world population uses medicinal plant for the treatment of disease and in African countries, this rate is much higher. It has been estimated that up to 90% of the population in developing countries rely on the use of medicinal plants to help meet their primary health care need (Segismundo *et al.*, 2008).

At least 25% of drugs used in modern pharmacopoeia are derived from plant while many are synthetic analogue built based on prototype compounds isolated from plants (Singh *et al.*, 2012).Medicinal plants contain combinations of several chemical compounds having multiple biological activities. Medicinal plants are increasingly becoming the subject of extensive studies worldwidefor their active therapeutic principles (Monira *et al.*, 2012; Shallangwa *et al.*, 2013a; 2013b). The rich wealth of plant kingdom can represent a novel source of newer compounds with significant pharmacological effects such as anticancer, anti-pneumonia, anti-diarrhea, anti-dysentery antiviral, antimicrobial include anti-inflammatory activities. The major merits of herbal medicine seem to be their perceived efficacy, low incidence of serious adverse effects and more affordable treatment (Iwu *et al.*, 1999).

Inflammation is a bodily response to injury, infection or destruction characterized by heat, redness, pain, swelling and disturbed functions (Shallangwa *et al.*, 2013b; Huo *et al.*, 2013). Inflammation is a normal protective response to tissue injury caused by physical trauma, noxious chemical or microbial agents. It is the body response to inactivate or destroy the invading organisms, to remove the irritants and set the stage for tissue repair. It is triggered by the release of chemical mediators' from injured tissue and migrating cells (Huo *et al.*, 2013; Lee *et al.*, 2013).Anti-inflammatory refers to the property of a substance or treatment that reduces inflammation. Anti-inflammatory drugs make up about half of analgesics, remedying pain by reducing inflammation as opposed to opioids, which affect the central nervous system (Gan *et al.*, 2010; Monira *et al.*, 2012; Shallangwa *et al.*, 2013b).

The most commonly used drug for management of inflammatory conditions are non-steroidal anti-inflammatory drugs (NSAIDs) and steroids which have several adverse effects especially gastric irritation leading to formation of gastric ulcers (Monira *et al.*, 2012; Shallangwa *et al.*, 2013b). One of the features of several non-steroidal anti-inflammatory drugs for example indomethacin, ibufenac, flufenamic acid and salicylic acid is their ability to prevent denaturation of heat treated bovine serum albumin (Umapathy *et al.*, 2010; Chandra *et al.*, 2012a; 2012b) at pathological pH (pH 6.2 – 6.5).

Some plants, vegetables and fruits are reported to be rich sources of antioxidants, such as vitamin A, vitamin C, Vitamin E, carotenoids, polyphenolic compounds, flavonoids and hydrolysable tannins, which can significantly destroy the free radicals, reactive oxygen species responsible for degenerative diseases or prevent free radical damage, thus reducing the risk of chronic diseases (Umapathy *et al.*, 2010). These plants include *Ocimum basilicum, Cinnamomum cassia, Ginkgo biloba, Camellia sinensis, Aloe vera, Malus domestica, hibiscus sabdariffa, Prunella vulgaris, Thymus vulgaris, Moringa oleifera* and quite a number of others.

Moringa oleifera Lam, a flowering plant, is one of the fourteen species of the genus *Moringa* of the *Moringaceae* family, found in Nigeria, India, Pakistan, Afghanistan, Bangladesh and in other parts of Africa and Latin America (Araújo *et al.,* 2012; Adejumo *et al.,* 2012). *Moringa oleifera* is widely distributed and naturalized in different climate andcould be found even in the harshest and driest of soils (Adejumo *et al.,* 2012). In some parts of the world *Moringa oleifera* is referred to as the 'drumstick tree', 'tree for purifying' or the 'horse radish tree' (Anwar *et al.,* 2007). *Moringa oleifera* leaves are reported to be a rich source of β-carotene, protein, vitamin C, calcium and potassium and act as a good source of natural antioxidants; and thus can enhance the shelf-life of fat containing foods due to the presence of various types of compounds that possess antioxidant properties, such as ascorbic acid, flavonoids, phenolics and carotenoids (Anwar *et al.,* 2007). Studies focusing on the chemical composition of this plant have identified many bioactive substances that may confer diverse pharmacological properties to the preparations obtained from its leaves, root (Aney*et al.,* 2009; Goyal *et al.,* 2007; Anwar *et al.,* 2007), bark (Aney *et al.,* 2009; Goyal*et al.,* 2007; Anwar*et al.,* 2007), gum (Aney *et al.,* 2009; Goyal *et al.,* 2007), fruit (Aney *et al.,* 2009; Anwar *et al.,* 2007), flowers (Adejumo *et al.,* 2012; Aney *et al.,* 2009; Anwar*et al.,* 2007), seeds andseed oil(Araújo *et al.,* 2012; Adejumo *et al.,* 2012; Minaiyan *et al.,* 2014).

Thymus vulgaris (Thyme) is aperennial, aromatic herb native toAsia, the Mediterranean regionand Europe, belongs to the *Lamiacea* family (Swayeh *et al.,* 2014; Rodrigues *et al.,* 2015). *Thymus vulgaris* is now widely cultivated in many parts of the world and is used as spice, tea and herbal drugs (Abd El Kader *et al.,* 2012; Hamzawy *et al.,* 2012; Swayeh *et al.,* 2014; Rodrigues *et al.,* 2015). *Thymus vulgaris* possess various beneficial effects, such asstimulant for the entire circulatory system, antiseptic, antimicrobial, antibacterial, anti-thelmintic, insecticidal, antifungal, antiviral, antioxidant, activities and, isalso effective in the treatment of depression and mood changes (Vale-Silva *et al.,* 2010; Abd El Kader *et al.,* 2012; Hamzawy *et al.,* 2012; Miguel *et al.,* 2012; Zuzarte *et al.,* 2013; Swayeh *et al.,* 2014; Rodrigues *et al.,* 2015). Thyme oils are also used in flavor and food industries, mainly in the manufacture of perfumes and cosmetics, or for flavoring chocolates, toothpastes, mouthwashes, and cough medicines (Vale-Silva *et al.,* 2010; Miguel *et al.,* 2012; Rodrigues *et al.,* 2015). All these activities are related to the high content of phenolic compounds, with special emphasis in thymol and carvacrol (Alizadeh, 2013; Silveira *et al.,* 2014; Rodrigues *et al.,* 2015).

This study is carried out to evaluate the total phenolic and flavonoid contents, and compare the possible anti-inflammatory effects of ethanol extracts of *Moringa oleifera* and *Thymus vulgaris* against the denaturation of protein *in vitro*.

Materials and Methods

The method used for assessing the anti-denaturation effects of natural products with anti-inflammatory properties is simple and inexpensive, as has been reported by several authors (Jagtap *et al.,* 2011; Shallangwa *et al.,* 2013a; 2013b; Shallangwa *et al.,* 2015).

Chemicals and drugs

The reference drug used for this work, IbuprofenPerrigo brand (800 mg per tablet) was purchased from Beautiful Pharmaceutical Drug store, opposite North-Gate, Ahmadu Bello University, Samaru-Zaria. Other reagents (hydrochloric acid, sodium dihydrogenphosphate, disodiumhydrogenphosphate, sodium chloride, glycerol) used were of analytical grades from BDH, M&B, Sigma or, Fluka.

Plant materials

Moringa oleifera leaves were obtained from a farm located in Kura LocalGovernment Area of Kano State, Nigeria; while *Thymus vulgaris* leaves were collected from Tiger Foods Limited, located in Awada Layout, Obosi, Anambra State, Nigeria. Both plants leaves were identified by a plant taxonomist, Mr. Namadi Sanusi at the Department of Botany, Faculty of Sciences, Ahmadu Bello University, Zaria, Kaduna state, Nigeria, where voucher specimen (*Moringa oleifera*, v/no: 571) and (*Thymus vulgaris*,v/no: 1933) were deposited .

Preparation of extracts

The fresh *Moringa oleifera* and *Thymus vulgaris* leavesmaterials were washed and chopped into bits and air-dried at room temperature for 7 days. The respective dried samples were separately ground in a laboratory grinder until smooth powders were obtained in order to ensure high surface area for increased diffusion. 50.00 g of each crushed plant materials were extracted with 80% ethanol by cool extraction for one week with constant shaking. The extracts were filteredusing 110 mm Filpap filter paper and allowed to dry in an open dish on a water bathcontrolled at 80^{o}C till constant weight was obtained. The percentage yield of each of plant was calculated. The dry extracts were kept in a vacuum desiccator until when ready for use (Shallangwa *et al.,* 2013b).

Estimation of total phenolic compounds and flavonoids

For the determination of total phenolic and flavonoid contents, stock solutions of gallic acid and rutin were prepared from which various concentrations (5, 10, 15, 20, 25, 30 µg/mL) used for plotting calibration curves were prepared by serial dilution. The amount of total phenolics in extracts was determined according to the Folin-Ciocalteu procedure (Kahkonen *et al.,* 1965; Shallangwa *et al.,* 2013b). Samples (200 µL, three replicates) were introduced into test tubes; 1.0 mL of Folin-Ciocalteu's reagent and 0.8 mL of sodium carbonate (7.5%) were added. The tubes were mixed and allowed to stand for 30 min. Absorption at 765 nm was measured on Helios Gamma UV-vis spectrophotometer, UVG-101505. The total phenolic content was expressed as gallic acid equivalents (GAE) in micrograms per milligram dry extract material (Shallangwa *et al.,* 2015); while the total flavonoid content of crude extract was determined by the aluminium chloride colorimetric method (Chang *et al.,* 2002; Shallangwa *et al.,* 2013b). 50 µL of crude extract (1 µg/mL ethanol) were made up to 1 mL with methanol, mixed with 4 mL of distilled water and then 0.3 mL of 5% $NaNO_2$ solution; 0.3 mL of 10% $AlCl_3$ solution was added after 5 min of incubation, and the mixture was allowed to stand for 6 min. Then, 2 mL of 1 moldm^{-3} NaOH solution were added, and the final volume of the mixture was brought to 10 mL with doubly distilled water. The mixture was allowed to stand for 15 min, and absorbance was measured at 510 nm. The total flavonoid content was calculated from a calibration curve, and the result was expressed as rutin equivalents (RE) in micrograms per milligram dry extract material. (Shallangwa *et al*., 2015).

In-vitro anti-inflammatory activity bioassay

The screening for anti-inflammatory activity was carried out according to a modification of the *in vitro* protein

denaturation bioassay methods described by Shallangwa *et al.* (2015), Jagtap *et al.* (2011) and and Sakat *et al.* (2010). Anti-inflammatory bioassay *in vitro* consist of reaction mixture (5 mL) which are 0.2 mL of egg albumin (from fresh hen's egg), 2.8 mL of phosphate buffered saline (PBS, pH 6.4) and 2 mL of varying concentrations of *Moringa oleifera* extract (of final concentrations 50, 100, 200, 400, 800, 1600 µg/mL, respectively). Similar compositions of 0.2 mL of egg albumin (from fresh hen's egg), 2.8 mL of phosphate buffered saline (PBS, pH 6.4) weremade where no drug or test sample was added but only varying amounts of doubly distilled water to serve as controlfor the respective runs. Then the mixtures were incubated at (37±2ºC) in Corsair Heating & Catering Limited incubator for 15 min. Denaturation was induced by keeping the reaction mixtures at 60±2ºC in water bath for 10 min. After cooling, the turbidity was measured at 660 nm (using SHIMADZU, UV 1800 Spectrophotometer). Percentage of inhibition of denaturation was calculated from control where no drug was added. Each experiment was done in triplicate and the average taken. Similar procedures adopted for the *Moringa oleifera* extractwere applied to*Thymus vulgaris* extracts. For the 1:1 blend, the anti-inflammatory bioassay *in vitro* reaction mixture (5 mL) consisted of 0.2 mL of egg albumin (from fresh hen's egg), 2.8 mL of phosphate buffered saline (PBS, pH 6.4) and 1 mL of varying concentrations of each of the plant extracts, *Moringa oleifera* and *Thymus vulgaris* (of final concentrations 50, 100, 200, 400, 800, 1600 µg/mL, respectively). The Ibuprofen tablet at the final concentrations of 50, 100, 200, 400, 800, 1600 µg/mL, respectively were also used as reference drug and treated similarlyas the *Moringa oleifera* and *Thymus vulgaris* extracts for determination of absorbance. Each experiment was done in triplicate and the average taken. The percentage inhibition of denaturation was calculated by using following formula (Jagtap *et al.*, 2011; Chandra *et al.*, 2012b):

$$\%\ Inhibition = 100x\left(V_t / V_c - 1\right)$$

Where

V_t= mean absorbance of test sample.

V_c= mean absorbance of control.

Statistical analysis

All the pharmacological test data are expressed as the Mean±Standard Error of the Mean (SEM). The 50% Inhibitory Concentrations (IC_{50}) expressed as 50% Effective Concentrations (EC_{50}) were determined at 95% Confidence Intervals after logarithmic transformation of the concentration-response (three parameters) curve using GraphPad Prism 6.01 software. The Efficiency Index (EI) was expressed as EC_{50}/E_{max}.

Results and Discussion

The overall extraction yield of *Moringa oleifera and Thymus vulgaris* were 33.59% and 23.79% dry weight materials. The study also revealed that the extracts were rich in phenolic and flavonoids. The results obtained for this study are in agreement with previous studies done by Vongsak *et al.* (2013) for *Moringa oleifera*; and Alizadeh (2013), Sabetsarvestani *et al.* (2013) for *Thymus vulgaris*. Total polyphenols of the extract of *Moringa oleifera and Thymus vulgaris* were estimated by the Folin-Ciocalteu method, calculated from a calibration curve (R^2= 0.9939), as 8.96±2.41 µg of GAE/mg and 20.86±1.66 µg of GAE/mg, respectively, dry extract weight. The total flavonoid contents of the two plants ethanolic extracts were calculated from a calibration curve (R^2= 0.9568) and are as presented in Table1. It has been reported widely that phenolic and flavonoids content show significant antioxidant action on human health (Sakat *et al.*, 2010; Jagtap *et al.*, 2011; Alizadeh, 2013; Vongsak *et al.*, 2013; Sabetsarvestani *et al.*, 2013; Shallangwa *et al.*, 2015). The polyphenol compounds like flavonoids, phenolic acids and tannins are known to have an ideal chemical structure for effective free radical-scavenging activities that have shown to be more effective as antioxidants *in vitro* than vitamins E and C. This fact has been established by many researches (Apak *et al.*, 2004; Kim and Lee, 2004). Furthermore, a number of previous studies have demonstrated a strong correlation between phenolic content, antioxidant properties and anti-inflammatory properties(Gan *et al.*, 2010; Umapathy *et al.*, 2010; Sakat *et al.*, 2010; Jagtap *et al.*, 2011; Chandra*et al.*, 2012a, 2012b; Shallangwa *et al.*,2013a, 2013b, 2015).

Table 1: Extraction yield, total polyphenols and total flavonoids contents of extracts from *Moringa oleifera* and *Thymus vulgaris*

Parameter	*Moringa oleifera*	*Thymus vulgaris*
Total yield (%W/W)	33.59	23.79
Total polyphenols contents (µg GAE/mg dry extract weight)	8.96±2.41	20.86±1.66
Total flavonoids contents (µg RE/mg dry extract weight)	5.82±1.38	8.26±1.28

GAE = Gallic acid equivalents; **RE** = Rutin equivalents

In the present study, the evaluation of anti-inflammatory effects was undertaken using the effect of *Moringa oleifera* and *Thymus vulgaris* extracts on protein denaturation. Denaturation of proteins is well documented and is caused by inflammation process, mostly in conditions like arthritis (Gan *et al.*, 2010; Umapathy *et al.*, 2010; Sakat *et al.*, 2010; Jagtap *et al.*, 2011; Chandra*et al.*, 2012a, 2012b; Shallangwa *et al.*,2013a, 2013b, 2015). Therefore, using agents that can prevent protein denaturation would be worthwhile for anti-inflammatory drug development. Several anti-inflammatory drugs have shown dose dependent ability to inhibit heat induced protein denaturation (Umapathy *et al.*, 2010; Sakat *et al.*, 2010; Jagtap*et al.*, 2011; Shallangwa *et al.*, 2015). Protection or inhibitory effect against protein denaturation, which is the main mechanism of action of NSAIDS, plays an important role in the anti-inflammatory activity of NSAIDS (Shallangwa *et al.*, 2013a, 2013b). The use of *in vitro* methods to study anti-inflammatory activities has its own advantages compared to using animals in experimental pharmacological research, because it addresses the ethical issues and the lack of rationale for the use of live animals when other suitable methods could be employed (Shallangwa *et al.*, 2015).

The ability of *Moringa oleifera* and *Thymus vulgaris* extracts to inhibit protein denaturation may contribute to their anti-inflammatory properties. In the present investigation, the *in vitro* anti-inflammatory effect of *Moringa oleifera* and *Thymus vulgaris* extracts, including a 1:1 blend of their extracts, were evaluated against denaturation of egg albumin. The results are as presented in Table 2.

The study showed a concentration-dependent inhibition of protein (egg albumin) denaturation by *Moringa oleifera*, *Thymus vulgaris* and the 1:1 blend of the two extracts, within the concentration ranges of 50.0 to 1600.0 µg/mL.

The reference drug, Ibuprofen, also exhibited concentration-dependent inhibition of protein denaturation. Table 2 showed that at low concentrations (50 µg/mL), the *Moringa oleifera* was more effective than the Ibuprofen standard drug, followed by *Thymus vulgaris* extract, and then the1:1 blend of extracts. At the concentration of 100 µg/mL, the trend observed was *Moringa oleifera* > *Thymus vulgaris* > Ibuprofen > extracts blend. As the concentration increased to the range of 200-800 µg/mL there was another slight change in the trend of percentage inhibition of denaturation: *Moringa oleifera* > *Thymus vulgaris* > *extracts blend* > Ibuprofenin preventing denaturation. But at higher concentration of 1600 µg/mL, another trend was observed yet, with the *Moringa oleifera* extract (507.6±2.2) showing that it is more effective than the 1:1 blend of extracts (383.3±3.0) in inhibiting denaturation of protein, followed by the *Thymus vulgaris* extract (322.6±0.8) and Ibuprofen (271.6±8.9), in that order, respectively. The study showed that within the concentration range of 200-1600 µg/mL, both extracts and their 1:1 blend were better in inhibiting protein denaturation than the reference drug, Ibuprofen.

Table 2: Anti-inflammatory data on *Moringa oleifera, Thymus vulgaris*, extracts blend of *Moringa oleifera* and *Thymus vulgaris* (1:1) and, Ibuprofen

Concentration (µg/mL)	*M. oleifera* (% inhibition)	*T. vulgaris* (% inhibition)	1:1 blend of *M. oleifera and T. vulgaris* (% inhibition)	Ibuprofen (% inhibition)
Control	--	--	--	--
1600	507.6±2.2	322.6±0.8	383.3±3.0	271.6±8.9
800	353.5±0.8	295.7±0.5	271.7±0.0	143.0±10.2
400	269.8±1.9	268.8±1.6	219.3±3.5	115.9±7.7
200	257.3±4.1	228.9±0.0	171.2±1.4	109.3±4.5
100	239.1±2.7	132.9±0.3	81.65±2.3	82.5±5.5
50	116.0±1.0	57.06±2.7	36.44±10.5	41.7±13.1

Values are expressed as SEM of 3 readings

Table 3: EC_{50} values for*Moringa oleifera, H. sabdariffa,* extracts blend and Ibuprofen

	Moringa oleifera	*Thymus vulgaris*	Extracts blend 1:1 (*M. oleifera/T. vulgaris*)	Ibuprofen
IC_{50} (µg/mL	215.9±29.8	144.9±1.1	441.6±8.6	1599±337

Values are expressed as SEM of 3 readings

The extract concentration for 50% inhibition expressed as effective concentration (EC_{50}) was determined by the dose-response curve using GraphPad Prism 6.01 software and values are as presented in Table 3. The EC_{50} is defined as the concentration sufficient to obtain 50% of a maximum inhibition of protein denaturation. In this study the EC_{50} values showed that the *Thymus vulgaris* extract is more effective than the *Moringa oleifera* extract, followed by the extracts blend, with the reference drug, Ibuprofen, being the least effective. The EC_{50} for Ibuprofen appeared todiffer considerably from the rest and therefore, there was a strong motivation tocharacterize it as anoutlier. Hence, the EC_{50} values in Table 3 calculated at 95% confidence level using the GraphPad Prism 6.01 software were subjected to the Dixon's *Q* testfor the rejection of grossly deviant values or outliers (Rorabacher, 1991). The statistic experimental Q-value (Q_{exp}) is 0.796, whilecritical Q-value (Q_{crit}) is 0.829, for N = 4 at 95% (α = 0.05) confidence level (Rorabacher, 1991). Since$Q_{exp} < Q_{crit}$, then the suspected value is accepted and retained.

Conclusion

One of the features of several non-steroidal anti-inflammatory drugs is their ability to inhibit or prevent denaturation of proteinas have been reported by several authors. From the results of this study, it can be concluded that *Moringa oleifera and Thymus vulgaris*, including their 1:1 blend, possessed marked anti-inflammatory effects as they can limit the denaturation of protein process *in vitro*. The result also showed that the two extracts and their blend are better than the reference drug in preventing denaturation at higher concentrations. This can contribute to the validation of the anti-inflammatory activity of these plants and may provide some evidence for their folk uses separately and in combinations. These results also provide the motivation for further planning of clinical nutrition and anti-inflammatory research studies on bioactive compounds of the plants and development of anti-inflammatory products.

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