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TLC SEPARATION AND ANTI-INFLAMMATORY ACTIVITY OF FLAVONOIDS OF *CISSUS INTEGRIFOLIA*: A TRADITIONAL VEGETABLE USED AS RELISH IN RURAL ZIMBABWE

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ABSTRACT

The study was concerned with evaluating the anti-inflammatory activity of flavonoids from *Cissus integrifolia*, a wild edible plant commonly consumed as relish in Zimbabwe. Thin layer chromatography was used to isolate flavonoids from ethanol and ethylacetate extracts of the plant. Ethanol and ethylacetate extracts gave three spots each which could be positively identified as flavonoids after spraying with 1% ethanolic aluminium chloride and viewing under ultraviolet light at 365 nm. The flavonoids were scratched, dissolved in the respective solvents, filtered, recovered on the rotor vapour and used to make flavonoid concentrations in the range 200 to 800 mg/L. Anti-inflammatory activity of the flavonoids was evaluated by the inhibition of egg albumin denaturation and NO radical scavenging assays and compared with indomethacin and quercetin as standards. Flavonoids of ethyl acetate extract of *Cissus integrifolia* exhibited anti-inflammatory activity that ranged from 34.11 to 67.68% and 16.75 to 45.14% for egg albumin inhibition and NO radical scavenging assays, respectively. On the other hand, ethanol extract flavonoids exhibited inhibition of protein denaturation that ranged from 27.03 to 64.21% and NO radical scavenging activity that ranged from 49.42 to 68.04%. Generally, flavonoids isolated from ethyl acetate extract are better protein denaturation inhibitors than they are NO radical scavengers, whereas, those from ethanol extract are better NO radical scavengers than they are inhibitors of protein denaturation. This implies the flavonoids extracted by the different solvents have different anti-inflammatory mechanisms.

Therefore, *Cissus integrifolia* is a promising nutraceutical and potential source of anti-inflammatory agents.

KEYWORDS: Anti-inflammatory activity, egg albumin assay, NO radical scavenging assay, *Cissus integrifolia*, thin layer chromatography.

INTRODUCTION

Inflammation is a pathophysiological condition which is a consequence of the body's response to infection, trauma, injury to name a few. It is initiated by the body as a defence reaction to eradicate or reduce the spread of harmful agents.[1] The condition can be classified as acute or chronic, with the former characterised by enhanced vascular permeability, capillary infiltration among others and the later by infiltration of mononuclear immune cells, macrophages, neutrophils.[2] Chronic inflammation has been known to cause diseases and conditions, including some cancers, rheumatoid arthritis, atherosclerosis, periodontitis, and hay fever.[3] Currently, nonsteroidal anti-inflammatory drugs (NSAIDs) are used for the treatment of inflammation-related diseases. However, these NSAIDs have been reported to cause side effects such as gastrointestinal bleeding, ulcers, hyperglycemia, hypertension among others.[4] In addition, there is recurrence of the symptoms with discontinued use[4] and also the drugs are expensive beyond the reach of many especially in developing countries. This may have contributed to the current shift towards traditional remedies for the treatment of inflammatory diseases by a large proportion of the world. There has been a surge in the presence of traditional remedies in markets across the world.[5] Natural remedies are generally considered safe with little or no side effects. Research has also shifted towards the search for nutraceuticals, which would provide nutrients and at the same time tackling disease conditions.

Flavonoids are among the many different groups of natural products. They are phytochemicals of the benzo-γ-pyrone derivatives that are widely distributed in the plant kingdom and are classified as chalcones, flavan-3-ols, flavanones, flavones, flavonols, isoflavones and biflavonoids.[6] Flavonoids have been referred to as nature's tender drugs with numerous biological or pharmacological properties such as anticancer, antiviral, antimicrobial, immunomodulatory and anti-inflammatory activities.[7] Anti-inflammatory ability of flavonoids has long been used in Chinese medicine and the cosmetic industry.[6] Various researches have established that the numerous flavonoid molecules possess anti-inflammatory activity on animal models of inflammation.[6] It is therefore important to

continuously evaluate the anti-inflammatory activity of flavonoids so as to establish anti-inflammatory activity mechanisms as well as developing new class of anti-inflammatory drugs.[6]

Cissus integrifolia is one of the many indigenous edible plants that is consumed in rural Zimbabwe as relish. It is generally considered as an agricultural weed and is one of the plants collected from the wild and consumed in several African and Asian countries mainly as a vegetable. Many rural communities in tropical Africa use such vegetables as *Cissus integrifolia* to supplement their diet which is based on rain fed cultivation of staples such as cassava, maize, millet, sorghum. Research done in 2013[8] showed that leafy vegetables gathered as weeds or from the wild form part of the diet of many rural households in Shurugwi District, Zimbabwe. *Cissus integrifolia* belong to the *Vitaceae* plant family. The stem of *Cissus integrifolia* when cut exudes a clear, tasteless, edible thirst quenching sap.[9] The anti-inflammatory potential of *C. integrifolia* flavonoids has not been explored. The objective of this study was to explore the anti-inflammatory potential of flavonoids of *C. integrifolia.* Information would be important to nutritionists for possible application as a nutraceutical and those on the field of pharmaceutical research.

MATERIALS AND METHODS

Plant material

Fresh leaves and shoots of *C. integrifolia* were collected from velds and fields of Zaka district in Masvingo Province of Zimbabwe during the month of March 2017. The samples were identified by a worker at the National Herbarium of Zimbabwe, at Harare Botanic Gardens. The samples are recorded under the flora of Zimbabwe: individual record number 60252: *Cissus integrifolia*. The samples were shade dried until constant weight was obtained. The samples were then ground to powder using a grinding machine (MODEL SM-450C). The powdered samples were stored in airtight containers in a dark cupboard until required for use in the study.

Standards and chemicals

Indomethacin, a drug used to treat inflammation, and used as the standard drug was procured from a local pharmacy. Quercetin (standard flavonoid) was procured from Sigma Aldrich (Sigma Aldrich, South Africa). All other chemicals were of analytical grade and were obtained from Sigma Aldrich (Germany) and Skylabs (South Africa). Double distilled water was used throughout the study.

Preparation of extract

Solvent extraction

About 20 g of powdered *C. integrifolia* sample were weighed on a METTLER TOLEDO digital analytical balance (AB204-S) and mixed with 100 ml of analytical grade absolute ethanol in 250 ml conical flasks. The samples were shaken for 24 h on a LABOTEC horizontal shaker. The samples were then filtered using WHATMAN No. 1 filter paper and placed in reagent bottles. The solvent extraction procedure was repeated twice more on the samples with shaking for a period of 30 minutes and the collected filtrates were combined and concentrated under reduced pressure on a rotor vapour at 40 ^{0}C. After complete drying the extracts were redissolved in 3 ml acetone and air dried. The yield was weighed and percentage yield was calculated. The solvent extraction process was repeated using ethyl acetate as solvent and percentage yield also calculated.

Analytical Thin Layer Chromatography

The *C. integrifolia* ethanol and ethylacetate extracts were each subjected to thin layer chromatography (TLC). As per conventional one dimensional ascending method, pre-coated silica gel TLC plates 10 x 1.5 cm (Merck, Germany) were cut from larger plates with scissors. The cut plates were then activated by heating at 100 ^{0}C for 10 min, and were allowed to cool to room temperature. A line, 1 cm above and parallel to silica gel TLC plate bottom, was drawn using a ruler and soft pencil and a small spot of solution containing the extract was applied on the pencil line using a thin capillary tube. The solvent was allowed to completely evaporate.

A previous method[10] was used with modifications. The spotted plates were placed into a TLC development chamber and developed with different trial solvent systems to find the suitable mobile phase, and this was done after pre-saturation of the chamber with the mobile phase. Pre-saturation was done by pouring the solvent into the development chamber to a depth of less than one centimetre, placing a large piece of cut filter paper in the chamber and then closed and left for 10 min to let the solvent vapour ascend the filter paper and saturate the air in the chamber. The solvent front was allowed to travel on the spotted plates until 1 cm from the top end.

After TLC plate development the TLC plates were removed and the solvent front was marked using a soft pencil, air dried, sprayed with a fine spray of 1% ethanolic aluminium chloride solution, left to dry and then visualized under UV/Vis light at 365 nm. The colour of the spots

on the chromatograms were noted and the spots were marked and their retention factors (R_f values) calculated using the formula:

$$R_f = \frac{\text{Distance travelled by solute}}{\text{Distance travelled by solvent front}}$$

The R_f values and the colour of the spots were recorded. R_f values of the spots which indicated the presence of flavonoids were also noted. The resultant chromatograms were captured on camera. The hexane:ethyl acetate:methanol:acetic acid (9:1:0.2:0.1, v/v/v/v) produced the optimum separation of the phytochemicals for both the ethanol and ethyl acetate extracts.

Preparative Thin Layer Chromatography

Pre-coated thick silica gel on glass TLC plates measuring 20 x 20 cm were used. The solvent system that gave optimum separation of phytochemicals in analytical TLC, hexane:ethyl acetate:methanol:acetic acid (9:1:0.2: 0.1, v/v/v/v), was used in the preparative TLC. A line was marked using a soft pencil and ruler at a distance of 1.5 cm from the edge. Each of the extracts, ethanol and ethyl acetate, was deposited as a concentrated band on the starting line of the TLC plates and allowed to dry. The plates, with dried samples were gently lowered into the development tank, closed and left to develop. The plates were removed when the solvent front had travelled three quarters of the plates' length. The position of the solvent front was immediately marked with a soft pencil. The retention factor values of the different bands were then calculated using the equation:

$$R_f = \frac{\text{Distance travelled by solute}}{\text{Distance travelled by solvent front}}$$

The bands that tested positive for the flavonoids in the analytical TLC were scratched off separately and mixed with 5 ml of their original extraction solvents. They were allowed to stand for 10 min and then filtered on WHATMAN No. 1 filter paper and the flavonoids collected in a glass vials. The filtrates were evaporated to dryness and the yield of flavonoids determined.

Preparation of flavonoid test samples and standards

The various separated flavonoids were serially diluted with double distilled water to make concentrations from 200-800 mg/L of the ethanol and ethylacetate plants extracts of *C. integrifolia*.

For quercetin standard, 25 mg was dissolved in 25 ml of methanol to make 1000 mg/L solution. The 1000 mg/L solution was serially diluted to give solutions of concentration 200, 400, 600, 800 mg/L. Similarly, 25 mg of indomethacin were also dissolved in 25 ml of methanol making 1000 mg/L stock solution. The stock solution was then serially diluted to give solutions of concentration 200, 400, 600, 800 mg/L.

Preparation of phosphate buffer saline

For preparation of phosphate buffer saline (PBS), anhydrous sodium dihydrogen orthophosphate (2.7250 g), disodium hydrogen orthophosphate (0.8000 g) and sodium chloride (22.5000 g) were weighed on a balance (METTLE TOLEDO digital analytical balance AB204-S) and dissolved in distilled water. The solution was put in a 250 mL volumetric flask and diluted to volume. The pH of the buffer was adjusted to 7.4 using 0.1 N HCI or NaOH.

Inhibition of heat induced egg albumin denaturation assay

The anti-inflammatory activity of flavonoids of *C. integrifolia* ethanol or ethylacetate and of quercetin and indomethacin standards were determined *in vitro* against denaturation of protein (albumin).[11] A reaction mixture of 5 mL was made by mixing 0.2 ml of 1% egg albumin solution, 2 ml of different concentrations of the various separated flavonoids or standard and 2.8 ml of PBS (pH 7.4). Control was made by mixing 2 ml of double distilled water, 0.2 ml 1% egg albumin solution and 2.8 ml of PBS solution. The mixtures were then incubated at (37±2) ^{0}C for 30 min and heated in a water bath at (70±2) ^{0}C for 15 min. After cooling, the absorbance was measured at 280 nm by UV-Vis spectrophotometer (GENESYS10S UV-Vis) using double distilled water as blank.

Indomethacin and quercetin were used as reference drug and flavonoid, respectively. Percent inhibition of egg albumin denaturation was calculated from the equation:

$$\text{Percentage Inhibition} = \frac{\text{Absorbance of control} - \text{Absorbance of test sample}}{\text{Absorbance control}} \text{ x } 100\%$$

NO radical scavenging assay

The nitric oxide (NO) radical scavenging assay was done according to a previous method.[12] Griess reagent was prepared by mixing equal amounts of 1% sulphanilamide in 2.5% phosphoric acid and 0.1% naphthylene diamine dihydrochloride in 2.5% phosphoric acid immediately before use. A volume of 0.5 ml of 10 mM sodium nitroprusside in phosphate

buffered saline was mixed with 1 ml of the different concentrations of the various separated flavonoids (200-800 mg/L) and incubated at 25 ^{0}C for 180 min. The prepared flavonoid concentrations or standard were mixed with an equal volume of freshly prepared Griess reagent. Control samples without the flavonoid extracts or standard but with an equal volume of buffer were prepared in a similar manner. The absorbance was measured at 546 nm using a UV-Vis spectrophotometer (GENESYS10S UV-Vis) by using double distilled water as blank. The percentage inhibition of the extract or standard was calculated and recorded. The percentage nitrite radical scavenging activity of the sample extracts or standard were calculated using the formula:

$$\% \text{ Nitric oxide scavenged} = \frac{\text{Absorbance control} - \text{Absorbance of test}}{\text{Absorbance of control}} \times 100$$

Statistical Analysis

All results were expressed as the mean ± standard deviation. In order to determine the measurement reproducibility the inhibition of egg albumin denaturation and NO radical scavenging assays of each sample were measured three times. Statistical analysis was performed by one-way ANOVA using the SPSS statistical software. All analyses were performed at the 95% confidence level and a p-value less 0.05 was assumed as the statistically significant difference between the experimental points.

RESULTS AND DISCUSSION

Table 1 gives the yield of crude ethanol and ethyl acetate extracts of *C. integrifolia*. The obtained yield was higher for ethanol and shows that it would be viable to extract phytocompounds of medicinal interest from the plant species.

Table 1: Yield of crude ethanol and ethylacetate extracts of *C. integrifolia.*

Solvent	Plant	Mass weighed for solvent extraction/ g	Crude extract yield/g
Ethanol	*C. integrifolia*	20.0025	1.7264
Ethyl acetate	*C. integrifolia*	20.0037	1.4852

Analytical TLC of the ethanol and ethyl acetate extracts of *C. integrifolia*, after spraying with 1% ethanolic aluminium chloride, showed four and seven spots respectively, under UV light at 365 nm. This shows the presence of four different compounds in the ethanol and seven different compounds the ethyl acetate extracts of *C. integrifolia*. The R_f values of the different TLC spots for ethanol and ethyl acetate extracts have been calculated and recorded in Table 2.

Table 2: TLC flavonoid analysis of *C. integrifolia* extracts using 1% ethanolic $AlCl_3$ revealing agent.

Plant extract	R_f Value	Test for flavonoids	Mass of flavonoids/g
C. integrifolia ethanol extract	0.10 (yellow)	Positive	0.0456
	0.32	Negative	-
	0.39 (yellow)	Positive	0.0249
	0.95 (blue)	Positive	0.0082
C. integrifolia ethyl acetate extract	0.08(yellow)	Positive	0.0353
	0.31	Negative	-
	0.42 (yellow)	Positive	0.0210
	0.58	Negative	-
	0.77	Negative	-
	0.88	Negative	-
	0.96 (blue)	Positive	0.0231

Fig. 1: Shows a typical analytical TLC for ethyl acetate extract of *C. integrifolia*.

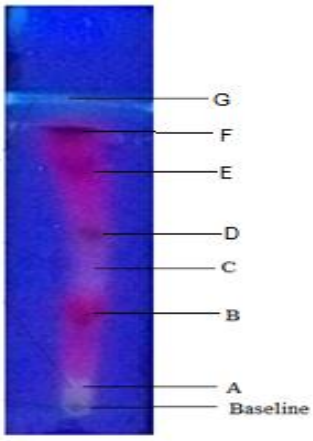

Fig. 1: A typical analytical TLC for *C. integrifolia* ethylacetate fraction showing seven spots with flavonoid spots A, C and G under UV light after spraying with 1% ethanolic $AlCl_3$

The results of the analytical TLC show that each of the two extracts has three different types of flavonoids. The sample bands showed yellow and blue colours under UV light indicating the presence of flavonoids in the plant extracts.

Orange or yellow colour is exhibited by flavonol glycosides according to previous reports.[13, 14] The blue spots are likely to be due to the presence of 5-deoxyisoflavones as well as 7,8-dihydroxyflavanones.[14,15] In addition, the blue spots could also be due to anthocyanidins-3-glycosides and anthocynidins-3,5-glycosides.[14] In this study a total of three flavonoids for ethanol and ethyl acetate extracts were found (Table 2). The results show

that ethanol extract of *C. integrifolia* had flavonoids with R_f values of 0.10, 0.39 and 0.95, while, ethyl acetate extract had flavonoids at R_f values at 0.08, 0.42 and 0.96. *C. integrifolia* ethanol extract (R_f = 0.10 and R_f = 0.39) could be related to morin (R_f = 0.13) and apigenin (R_f = 0.39) since same solvents save for methanol were used to make the mobile phase solvent composition in a previous report.[16]

Preparative TLC was made using the same mobile solvent system as that for analytical TLC, that is, hexane:ethyl acetate:methanol:acetic acid (9:1:0.2:0.1, v/v/v/v). The flavonoid containing bands were scrapped off using surgical blades, dissolved in the respective solvent and then filtered using a WHATMAN No. 42 filter paper. The filtered solutions were recovered on a rotor vapour and then dissolved again in respective solvents. Serial dilutions of the recovered solutions were made and their anti-inflammatory activity was tested using the inhibition of egg albumin denaturation and NO radical scavenging assays. Indomethacin (a standard anti-inflammatory drug) and quercetin (standard flavonoid) were used for comparison. For both extracts of the *C. integrifolia* only two of the three flavonoids were tested for their anti-inflammatory activity, that is, flavonoids at R_f = 0.08 and 0.96 for ethyl acetate extracts and R_f = 0.10 and 0.95 for ethanol extracts.

Fig. 2: Shows that percent protein denaturation inhibition increases steadily with increasing concentration of the ethyl acetate extract of the *C. integrifolia* flavonoid or standard.

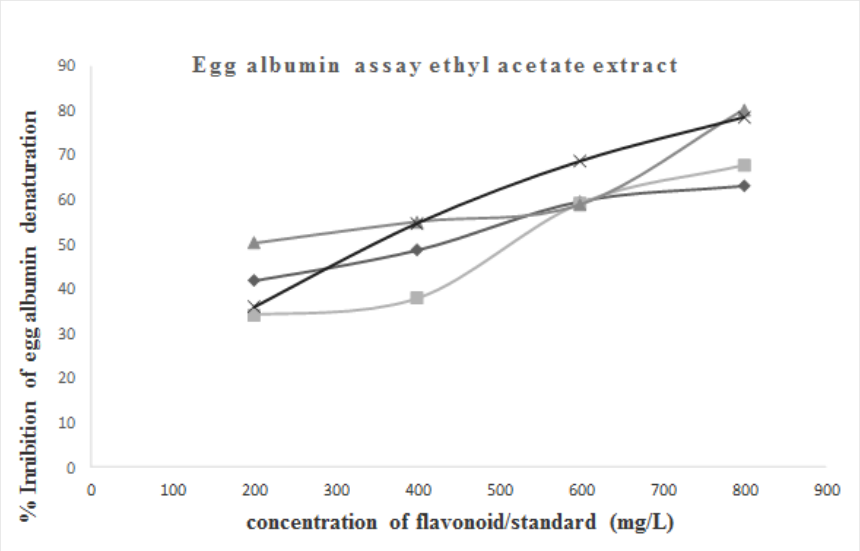

Fig. 2: Variation of inhibition of heat induced egg albumin denaturation with concentration of flavonoid/standard for ethyl acetate extract. ♦R_f = 0.96, ■R_f = 0.08, ▲Indomethacin, × Quercetin.

Indomethacin has higher protein inhibition (50.20 to 55.01%) than all the three flavonoids (R_f = 0.96; R_f = 0.08 and quercetin) in the 200-400 mg/L range, becoming steady (55.01 to 58.89%) in the 400-600 mg/L and then rising sharply (58.89 to 80.19%) in the 600-800mg/L range. *C. integrifolia* flavonoid (R_f = 0.96) had high inhibitory activity than quercetin standard at lower concentration (200-300 mg/L) but at higher concentration (300-800 mg/L) quercetin has superior inhibition of protein denaturation that surpasses even that of indomethacin (from 400 to close to 800 mg/L) (Fig. 2). Flavonoid at R_f = 0.08 has consistently lower protein denaturation inhibition (200-600 mg/L), however, above 600 mg/L the flavonoid has superior protein denaturation inhibition than flavonoid at R_f = 0.96. Statistical analysis was performed using one-way ANOVA with Tukey's post hoc tests, comparing percent inhibition of protein denaturation by *C. integrifolia* flavonoids (R_f 0.08 and 0.96), quercetin and indomethacin at a given concentration. The results show that at 200 mg/L, *C. integrifolia* flavonoid (R_f = 0.08) and quercetin percent inhibition of protein denaturation was not significantly different (p = 0.778). At 600 mg/L, that of indomethacin was not significantly different (p = 0.441) from the *C. integrifolia* flavonoid (R_f 0.96), that at R_f 0.08 (p = 0.922). There was also no significant difference (p = 0.778) between the two *C. integrifolia* flavonoids (Table 3).

Table 3: Egg albumin inhibition assay of flavonoids of ethyl acetate extract of *C. integrifolia*.

Egg Albumin Inhibition Assay				
Concentration (mg/L)	Ethyl acetate solvent		Indomethacin %Inhibition	Quercetin %Inhibition
	R_f = 0.96 % Inhibition	R_f = 0.08 %Inhibition		
200	41.71±0.39[a]	34.11±4.45[b]	50.20±0.35[c]	35.83±0.31[b]
400	48.58±2.61[a]	37.80±0.81[b]	55.01±0.52[c]	54.67±0.05[c]
600	59.57±0.25[a]	59.16±0.79[a]	58.89±0.63[a]	68.64±0.21[b]
800	63.00±0.46[a]	67.68±0.81[b]	80.19±0.15[c]	78.44±0.41[d]

Values in the same row with the same letter are not statistically significant according to Tukey's HSD post hoc test.

Fig. 3: Shows the same flavonoids (R_f 0.08 and 0.96) from the ethyl acetate extract of *C. integrifolia* in the NO radical scavenging assay.

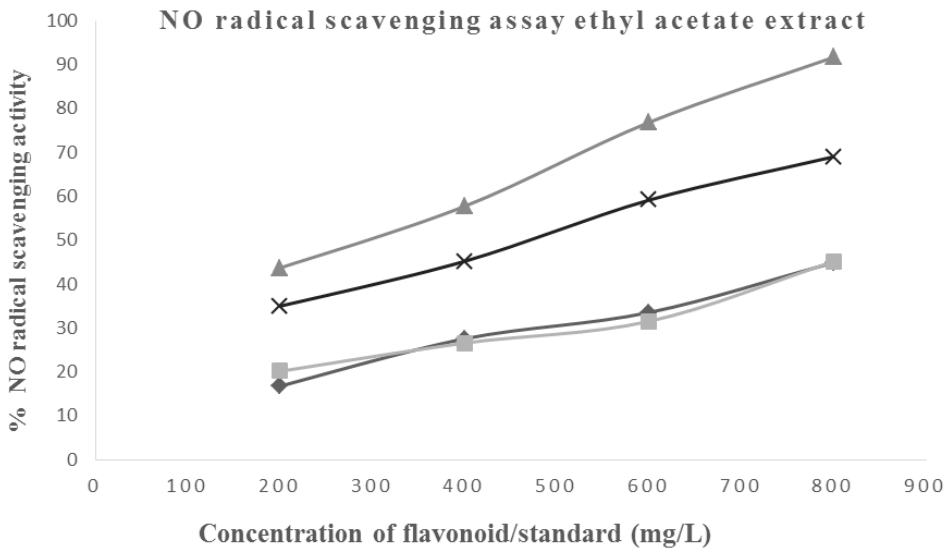

Fig. 3: Variation of NO radical scavenging activity with concentration of flavonoid/standard for ethyl acetate extract. ♦R_f = 0.96, ■R_f = 0.08, ▲Indomethacin, × Quercetin.

The results show that the standards (indomethacin and quercetin) have superior NO radical scavenging activity throughout the concentration range studied compared to the two *C. integrfolia* flavonoids. The two flavonoids have comparable NO radical scavenging activity with R_f 0.08 ranging from 20.13 to 45.14% and R_f 0.96 from 16.75 to 44.19%.

At 800 mg/L, the two flavonoids (R_f 0.08 and 0.96), have NO radical scavenging activity which was not statistically significant ($p = 0.783$). Although not tasted statistically, this can also be seen in the concentration between 300 and 400 mg/L (Fig. 3).

The two ethanol extract flavonoids (R_f = 0.10 and R_f = 0.96) of *C. integrifolia* were also tested for their anti-inflammatory activity using the egg albumin as well as the NO radical scavenging assays. Fig. 4 shows that indomethacin had higher inhibition of heat induced protein denaturation (50.20 to 55.01%) followed by *C. integrifolia* flavonoid (R_f = 0.10) (42.84 to 54.83%) and quercetin (35.83 to 54.67%) in the 200-400 mg/L concentration range.

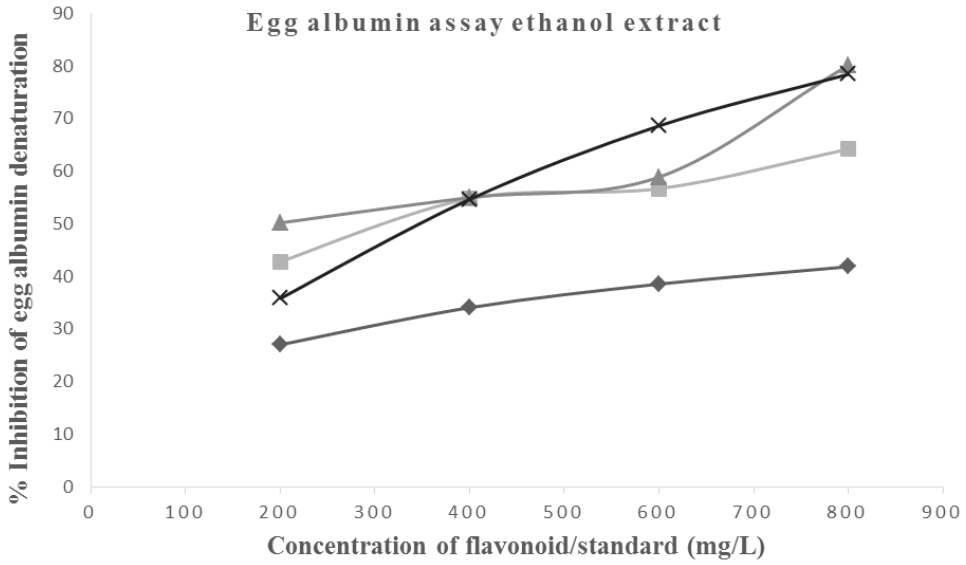

Fig. 4: Variation of inhibition of heat induced egg albumin denaturation with concentration of flavonoid/standard for ethanol extract. ♦R_f = 0.95, ■R_f = 0.10, ▲Indomethacin, × Quercetin.

At 400 mg/L the inhibition of protein denaturation of indomethacin (55.01%) was not significantly different (p = 0.973) from *C. integrifolia* flavonoid (R_f = 0.10) and also that of quercetin (54.67%) (p = 0.845). There was also no significant difference (p = 0.977) between the quercetin and *C. integrifolia* flavonoid R_f = 0.10 (Table 5).

Table 5: Egg albumin inhibition assay of flavonoids of ethanol extract of *C. integrifolia*.

Egg Albumin Inhibition Assay				
Concentration (mg/L)	**Ethanol solvent**		**Indomethacin %Inhibition**	**Quercetin %Inhibition**
	R_f = 0.95 % Inhibition	**R_f = 0.10 %Inhibition**		
200	27.03±0.61[a]	42.82±1.00[b]	50.20±0.35[c]	35.83±0.31[d]
400	34.07±0.74[a]	54.83±0.50[b]	55.01±0.52[b]	54.67±0.05[b]
600	38.55±0.23[a]	56.71±0.22[b]	58.89±0.63[c]	68.64±0.21[d]
800	41.86±0.92[a]	64.21±0.16[b]	80.19±0.15[c]	78.44±0.41[d]

Values in the same row with the same letter are not statistically significant according to Tukey's HSD post hoc test.

Above 400 and up to about 550 mg/L, a trend can clearly be seen for indomethacin and the *C. integrifolia* flavonoid (R_f = 0.10), showing that the two have the same inhibition of protein denaturation in this concentration range, however, that of indomethacin rises sharply above

600 mg/L. From 400 mg/L onwards quercetin exhibits superior inhibition than that of indomethacin and flavonoid of *C. integrifolia* (R_f = 0.10) (Fig. 4).

The *C. integrifolia* flavonoid at R_f = 0.96 has consistently lower inhibition of protein denaturation compared to the flavonoid at R_f = 0.10, quercetin and indomethacin throughout the concentration range studied (Fig. 4).

In the NO radical scavenging assay of flavonoids of the same *C. integrifolia* ethanol extract, the flavonoid at R_f = 0.10 exhibited almost constant inhibition of protein denaturation (65.46 to 68.04%) throughout the concentration range studied (200 to 800 mg/L) (Fig. 5).

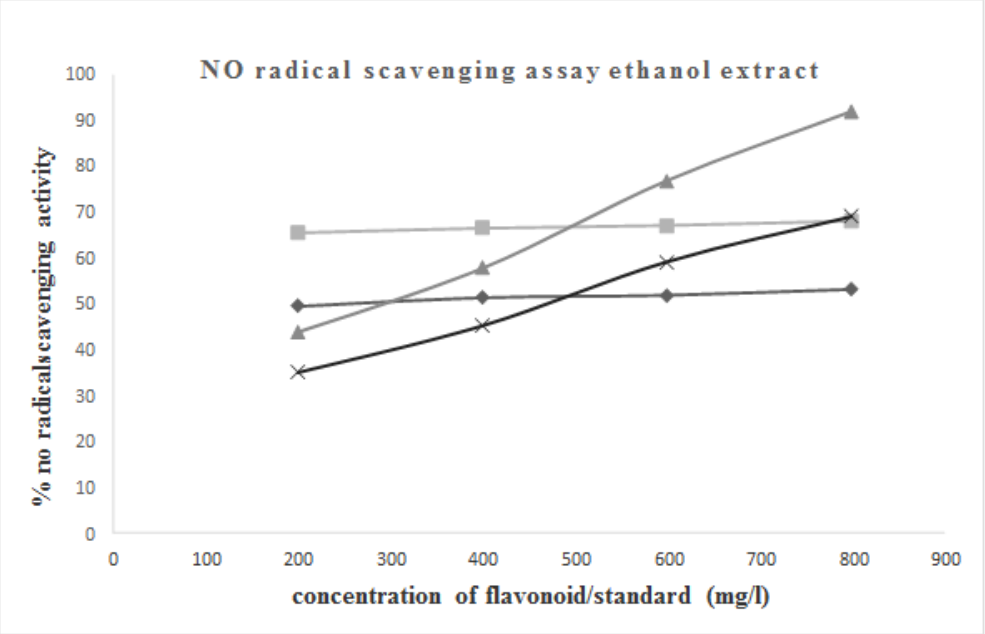

Fig. 5: Variation of NO radical scavenging activity with concentration of flavonoid/standard for ethanol extract. ♦R_f = 0.95, ■R_f = 0.10, ▲Indomethacin, × Quercetin.

The NO radical scavenging assay of this flavonoid (R_f = 0.10) was superior to all others in the range 200 to about 500 mg/L, this was followed by flavonoid at R_f = 0.96 which was also superior to quercetin and indomethacin in the range 200 to about 300 mg/L, but also exhibited almost constant inhibition (49.42 to 53.15%) throughout the concentration range studied (Figure 5). The NO radical scavenging activity exhibited by indomethacin increases steadily from 43.80% and surpasses that of *C. integrifolia* flavonoid (R_f = 0.96) around 300 mg/L and that of flavonoid at R_f = 0.10 at around 500 mg/L (Fig. 5). The NO radical scavenging activity of quercetin is consistently lower than that of all the others in the

concentration range 200 to about 500 mg/L where it surpasses that of R_f = 0.96 flavonoid, and eventually meets that of flavonoid R_f = 0.10 close to concentration of 800 mg/L.

Protein denaturation is a well-documented factor that is known to trigger inflammatory and arthritic conditions that include cancer, osteoarthritis and spondylitis among others.[17,18,19] Protein degeneration is the loss of secondary and tertiary structure of proteins as a result of external stress or factors such as high temperature, strong acid/base[19] which results in loss of biological function of the protein. As a result, inhibition of protein denaturation is vital in preventing inflammatory conditions. Flavonoids have been reported[20] to have the ability to chelate metal cations and therefore be able to protect protein membrane from denaturation. Proteins denatured by heat triggers delayed hypersensitivity which is associated with conditions such as rheumatoid arthritis, glomerulo-nephritis.[21] In the present study, flavonoids isolated by TLC from the ethanol and ethyl acetate extracts of the traditional relish *C. integrifolia* were seen to be effective in inhibition of protein (albumin) denaturation (Fig. 2 and 4). This suggests that *C. integrifolia* could be utilised as a nutraceutical or alternatively could be used as a source of flavonoids for further development to anti-inflammatory agents.
NO radical is produced in mammalian cells and is important for physiological processes such as destroying bacteria and viruses. Overproduction of NO, however, is associated with various ailments such as inflammation of airways in asthmatic patients.[22] NO radical is known to react with the superoxide ion forming peroxynitrite ($ONOO^-$)[23], a cytotoxic molecule whose protonated form, peroxynitrous acid (ONOOH) is a powerful oxidant.[24] Peroxynitrous acid forms an adduct with carbon dioxide in body fluids which causes oxidative damage to proteins in living systems. It is therefore important for anti-inflammatory agents to be present in living systems to neutralise excess NO radicals. Anti-inflammatory phytocompounds would compete with oxygen to react with the NO radical thus halting the formation of the damaging peroxynitrous acid. In this study TLC separated flavonoids of the ethanol and ethyl acetate extracts of *C. integrifolia* exhibited significant NO radical scavenging activity (Fig. 3 and 5). Therefore, when taken as relish the flavonoids of *C. integrifolia* could be of benefit by preventing nitrite formation hence prevent damages to biological molecules in living systems. Flavonoids have been reported before to exhibit anti-inflammatory activity due to their antioxidant properties.[25] Some anti-inflammatory drugs have been shown to have antioxidant and radical scavenging mechanism as part of their activity.[26, 27]

In this investigation a promising source of anti-inflammatory flavonoids has been identified in the rather not well investigated plant *C. integrifolia* which is used as a traditional vegetable in Zimbabwe and some parts of Africa. The anti-inflammatory activity of ethyl acetate and ethanol extracts of *C. integrifolia* were studied using the inhibition of albumin denaturation and NO radical scavenging assays. The results showed that the TLC isolated flavonoids exhibited significant anti-inflammatory activity by both assays in the concentration range studied. In addition, the results showed that flavonoids isolated from ethyl acetate extract of *C. integrifolia* (R_f = 0.96 and 0.08) exhibited inhibition of heat induced protein denaturation anti-inflammatory mechanism better than the NO radical scavenging mechanism *in vitro* in the entire concentration range (200 to 800 mg/L) studied. By contrast flavonoids isolated from ethanol extract of *C. integrifolia* (R_f = 0.95 and 0.10) exhibited NO radical scavenging anti-inflammatory mechanism *in vitro* better than the inhibition of protein denaturation mechanism throughout the concentration range studied. There is need to carry out additional studies to elucidate the structures of these flavonoids which may be useful in introducing new anti-inflammatory agents.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

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