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Anti-inflammatory and Antioxidant Activities of *Hunteria umbellata* Seed Fractions

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Abstract: Background: Decoction of *Hunteria umbellata* seeds is highly valued in African herbal medicine in the management of inflammation, diabetes and obesity. This study evaluates the anti-inflammatory and antioxidant activities of crude (HU) butanol (HU_b) and alkaloid (HU_{Af}) fractions of the water seed extract of *Hunteria umbellata*, in addition, to determining the phenolic content of HU and Hu_b. **Materials and methods:** The acute anti-inflammatory activity of 50 mg kg^{-1} of each of the seed fractions was evaluated in carrageenan-and formalin-induced oedematous Wistar rats. The fraction's antioxidant activities were evaluated using 1,1-Diphenyl-2-picrylhydrazyl (DPPH) free radical, superoxide anions and nitric oxide scavenging methods, in addition, to determining the phenolic contents of the fractions using standard procedures. **Results:** Results showed that HU, HU_b and HU_{Af} significantly ($p<0.05$ and $p<0.001$) inhibited carrageenan-and formalin-induced inflammation in the rats. Similarly, HU_b and HU_{Af} at 0.2-0.8 mg mL^{-1} exhibited significant ($p<0.05$ and $p<0.001$) DPPH free radical, superoxide anion and nitric oxide scavenging activities with the most significant effect recorded for HU_{Af}. Their proanthocyanidin contents were estimated to be 38.90±1.67 and 53.67±1.12 mg g^{-1} of dry extract, respectively while their total flavonoid contents were estimated to be 0.50±0.03 and 11.78±1.47 mg g^{-1} of dry extract, respectively. Also, their total phenolic contents were estimated to be 39.68±2.56 and 97.12±3.32 mg g^{-1} of dry extract. **Conclusion:** The anti-inflammatory activity of HU and its butanol fraction is attributed to its alkaloid content which was partly mediated via its anti-oxidant mechanism.

Key words: *Hunteria umbellata* (K. Schum.) Hallier f., aqueous seed fractions, anti-inflammatory activity, free radical scavenging/antioxidant mechanism

INTRODUCTION

Hunteria umbellata (K. Schum.) Hallier f. (family: Apocynaceae) is a West African glabrous tree which is known as Demouain (in French) (Boone, 2006) and Abeere (in Yoruba, Southwest Nigeria) (Ibeh *et al*., 2007; Adeneye and Adeyemi, 2009a). In African folk medicine, *Hunteria umbellata* plant has wide therapeutic applications including the local treatment of pain, swellings, infections, gastric ulcers, liver diseases, diabetes mellitus, obesity and management of labour at term (Ejimadu and Falodun, 2002; Falodun *et al*., 2006; Adeneye and Adeyemi, 2009a). In Germany, bitter tonics made from *Hunteria umbellata* are used for reducing high blood pressure and blood lipids (Boone, 2006). Recently, the antihyperglycaemic effects of the aqueous seed extract in different experimental models of diabetes (Adeneye and Adeyemi, 2009a, b; Igbe *et al*., 2009a) and its anti-obesity and anti-hyperlipidaemic effects (Adeneye *et al*., 2010a) were reported. Acute and chronic oral toxicity studies have equally shown the aqueous seed extract of the plant to be relatively safe in rats pre-treated with the extract (Adeneye *et al*., 2010b).

In African traditional medicine, water decoction of the pulp and dried seeds of *Hunteria umbellata* is reputed for the management of arthritic swellings (Igbe *et al*., 2009b). Recently, the antipyretic and analgesic effects of the fruit pulp extract of the plant were validated (Igbe *et al*., 2009b). Despite the historical and extensive folkloric use of the water infusion of the plant seed in the management of arthritic swelling, there is no evidence to back up this folkloric claim. This forms the basis of the current study

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which evaluates the anti-inflammatory effect and mechanism(s) of HU and its fractions in wistar rats. Also, bearing in mind the strong association between inflammation and ROS generation (Schreckinger *et al*., 2010), the current study evaluates the *in vitro* antioxidant activity of the aqueous seed extract of *Hunteria umbellata* (HU), its butan-1-ol fraction (HU_b) and its total alkaloid fraction (HU_{Af}) using 1,1-Diphenyl-2-picrylhydrazyl (DPPH) free radical, superoxide anion and nitric oxide scavenging methods. In addition, the polyphenol derivative contents of the extracts were also estimated using standard procedures.

MATERIALS AND METHODS

Collection of plant materials: Dried seeds of *Hunteria umbellata* were purchased from a retailer of herbal produce in Jakande Estate, Oke-Afa, Isolo, Lagos State, Nigeria, in December 2009. These were identified and authenticated by Mr. T.K. Odewo (Chief Superintendent of Taxonomy) at the Herbarium of the Department of Botany and Microbiology, Faculty of Science, University of Lagos, Akoka, Lagos, Nigeria. The seeds were de-coated of their light-brown coatings, gently rinsed in tap water and completely air-dried under room temperature (30±2°C) for 4 weeks protected from direct heat or sunlight. The seeds were kept in air-and water-tight container and stored in the refridgerator.

Aqueous extraction of hunteria umbellata seeds: One hundred gram of the dried seeds was pulverized to white-to-light brown fine powder using a domestic blender. Thirty gram of the fine powdered sample was macerated in 500 mL of distilled water in a 1 L Pyrex beaker and kept in the refrigerator at 4°C for 72 h. After 72 h, the homogenate was then continuously stirred for 6 h before it was rapidly filtered through a piece of clean white cloth. The filtrate was then completely dried to an aromatic, deep brown solid residue in an aerated oven preset at 40°C. The weight of the solid residue left behind was 23 g, giving a yield of 23.0% (w/w). The residue was stored in air-and moisture-tight container which was kept in a refrigerator. From this, a fresh stock was reconstituted in distilled water at a concentration of 100 mg mL^{-1}, whenever needed.

Solvent partitioning of HU: Thirty gram of the extract was suspended in 100 mL of distilled water. The solution was then transferred into 5 L burette before it was partitioned using between 1 to 1.5 L of different partitioning solvents (diethyl ether, chloroform, acetyl acetate and butan-1-ol) in the order of their increasing solubility gradients. The fraction obtained with each partitioning solvent was concentrated *in vacuo* using rotary evaporator (BÿUÿCHI Rotavapor® Model R-215, Switzerland) with Vacuum Module V-801 EasyVac®, Switzerland) set at a revolution of 70 rpm and a temperature of 35°C. The solid residue and the concentrate of each fraction were then transferred to an aerated oven preset at 35°C for complete dryness. The residues left after oven drying were then weighed. This procedure was repeated thrice and each residue was pooled together and stored in clean and dry, water and air-proof containers and preserved in the refrigerator until required for experimentation.

Determination of DPPH scavenging activity of HU and its fractions: The effect of HU, its butanol and alkaloid fractions was estimated using the method of Liyana-Pathiranan and Shadidi (2005). A solution of 0.135 mM 1,1-Diphenyl-2-picrylhydrazyl (DPPH) (Sigma Aldrich, St. Louis, USA) in methanol was prepared and 1.0 mL of this solution was mixed with 1.0 mL of methanol containing 0.2-1.0 mg of each extract. The reaction mixture was vortexed thoroughly and left in the dark at room temperature or 30 min. The absorbance of the mixture was measured spectrophotometrically at 517 nm. Ascorbic acid (Sigma Chemicals Co., St. Louis, USA) equally prepared at same concentration was used as the reference drug. The experiment was conducted in triplicate. The ability to scavenge DPPH radical was calculated by the following equation:

$$\text{DPPH radical scavenging activity (\%)} = \frac{(Abs_{control} - Abs_{sample})}{(Abs_{control})} \times 100$$

Where:

$Abs_{control}$ = Absorbance of DPPH radical + methanol

Abs_{sample} = Absorbance of DPPH radical + sample extract/ standard

Determination of superoxide anion and nitric oxide scavenging activities of HU and its fractions: Superoxide anion and nitric oxide scavenging activities of HU and its fractions were evaluated using the methods of McCord and Fridovich (1969) and Sreejayan and Rao (1997), respectively. In both assaying methods, quercetin was used as the standard drug.

Quantitative determination of polyphenolic contents of HU and Hu_b: The total flavonoids, proanthocyanidins and phenolic contents in HU and HU_b were determined using standard procedures described below:

Determination of total flavonoid content in HU and HU_b: Total flavonoids in HU and HU_b were estimated using the method of Ordonez *et al*. (2006) with a modification and as

adopted by Adedapo *et al.* (2008). To 1 mL of crude HU and HU_b, equivalent 1 mL of 2% aluminium chloride in ethanol solution was added. After 1 h of incubation at room temperature (28°C) for colour development, the absorbance was measured at 420 nm using ®Unico 2100 Spectrophotometer (United Products and Instruments Inc., Shanghai, China). A golden yellow colour indicated the presence of flavonoids. Total flavonoids contents were calculated as rutin hydrate (minimum 98%) (Sigma Chemicals Co., St. Louis, MO, USA) equivalent from the calibration curve $y = 44.77 x + 0.1036$, $R^2 = 0.9812$, where, x was the absorbance and y was the rutin equivalent (mg g^{-1}).

Determination of total proanthocyanidin contents in HU and Hu_b: Total proanthocyanidins (tannin) contents in HU and HU_b were estimated by method of Sun *et al.* (1998) and as adopted by Sofidiya *et al.* (2008). The 0.5 mL of 50 mg L^{-1} of the extract was mixed in 3 mL of 4% vanillin-methanol solution and 1.5 mL concentrated hydrochloric acid and the mixture was allowed to stand for 15 min at room temperature (28°C) for colour development. The absorbance was measured at 500 nm using ®Unico 2100 Spectrophotometer (United Products and Instruments Inc., Shanghai, China). Total proanthocyanidins contents were calculated as catechin hydrate (minimum 98%) (Sigma Chemicals Co., St. Louis, MO, USA) equivalent (mg g^{-1}) using the following equation based on the calibration curve: $y = 3.95 x-0.007$, $R^2 = 0.9218$ where, x was the absorbance and y was the catechin equivalent (mg g^{-1}).

Determination of total phenols in HU and Hu_b: Total phenol contents in HU and HU_b were determined by the modified Folin-Ciocalteu method of Wolfe *et al.* (2003) and as adopted by Sofidiya *et al.* (2008). An aliquot of each of HU and Hu_b was mixed with 2.5 mL Folin-Ciocalteu reagent (previously diluted with distilled water, 1:10 v/v) and 2 mL of (75 g L^{-1}) of sodium carbonate. The tubes were vortexed for 15 seconds and allowed to stand for 30 min at 40°C for colour development. Absorbance was measured 765 nm using ®Unico 2100 Spectrophotometer (United Products and Instruments Inc., Shanghai, China). Total phenolic content was expressed as mg g^{-1} rutin equivalent using the following equation based on the calibration curve: $y = 8.6 x + 0.2004$, $R^2 = 0.9814$, where x was the absorbance and y was the rutin equivalent (mg g^{-1}). The amount of total phenol was calculated as rutin equivalent from the calibration curve. The experiment was replicated thrice.

Extraction of alkaloid fraction (HU_{Af}) from HU: Ten gram of HU was suspended in 50 mL of 5% aqueous HCl acidified water and extracted four times with chloroform. The combined acid solution was carefully basified with sodium trioxocarbonate (IV) (Na_2CO_3) solution to pH 12 and then extracted three times with chloroform (150 mL) until the solution was almost colourless and the basic solution gave negative tests with alkaloid detecting reagents (Adegoke and Alo, 1986). The chloroform solution concentrated at the room temperature (32°C) to give a sweet-smelling, dark brown solid residue weighing 0.730 g (yield: 7.3%)This procedure was repeated three more times and the residues were pooled into a tight-capped container which was stored in the refrigerator until required for experiment.

Experimental animals: Fifty, young adult male Wistar rats (130-150 g) were obtained from the rat colony of the Animal House, College of Medicine, University of Lagos, Idi-Araba, Lagos, Nigeria, in the month of May, 2010, after ethical approval was obtained. Animals were housed in propylene cages, maintained under standard laboratory conditions and quarantined for 7 days to allow for acclimatization. Experimental animals were also handled and processed according to institutional international guidelines on the use of animals for experiment (United States National Institutes for Health, 1985).

***In vivo* anti-inflammatory evaluation of HU and its fractions**

Carrageenan-induced paw oedema: Twenty-five young adult male Wistar rats (130-150 g) were divided into five groups of five rats per group. In all the treatment groups, rats were orally pre-treated with the extract fractions 1 h before inducing acute inflammation with single sub-plantar injection of 0.1 mL of freshly prepared 0.5% (w/v) carrageenan suspension in normal saline using a modified method of Agbaje *et al.* (2008). The carrageenan solution in normal saline which was injected into the sub-plantar tissue of the left hind paw of the rat served as the tested while that of the right hind paw served as the control.

Treatment of rats is as described below:

Group I: 10 mL kg^{-1} of distilled water+0.1 mL of 0.5% carrageenan suspension

Group II: 100 mg kg^{-1} of aspirin+0.1 mL of 0.5% carrageenan suspension

Group III: 50 mg kg^{-1} of HU+0.1 mL of 0.5% carrageenan suspension

Group IV: 50 mg kg^{-1} of HU_b+0.1 mL of 0.5% carrageenan suspension

Group V: 50 mg kg^{-1} of HU_{Af}+0.1 mL of 0.5% carrageenan suspension

Aspirin was dissolved in distilled water and the extract fractions were dissolved in 10% Tween 20 in distilled water because of their incomplete solubility in distilled water. The paw thickness of animals in all groups was measured using vernier calipers before and 4 h after carrageenan injection. The volume of oedema for each rat was expressed as the difference in the diameter of the rat paw before and after injection of the carrageenan.

Formalin-induced paw oedema: In this model of experimental inflammation, the experimental procedure was the same as described above except that single dose of 0.2 mL of 2% formalin (v/v) in distilled water was injected into the rat paw to induce the inflammation.

In both models, the increases in paw thickness and percent inhibition were calculated using the formula below as adopted by Joseph *et al.* (2009):

$$P_t - P_0 = \frac{\text{Increase in paw thickness in control}}{\text{Treatment } P_c / P_t}$$

$$\text{Percentage inhibition}(\%) = \frac{P_c - P_T}{P_c} \times 100$$

where, P_t is paw thickness at time t, P_0 is initial paw thickness, P_c is the increase in paw thickness of the control group and P_T is the increase in paw thickness of the treatment groups.

Statistical analysis: Data were analysed using two-ways analysis of variance and the significance of the difference between the means were considered at $p<0.05$ and determined by student's t-test.

RESULTS

Solvent partitioning of HU: Partitioning of HU with diethyl ether, chloroform, acetyl acetate and butan-1-ol and the eventual oven-drying of the fractions yielded 0 g of diethyl ether fraction (%yield = 0%), 1 g of chloroform fraction (%yield = 3.3%), 2 g of acetyl acetate fraction (6.6%), 4 g of butan-1-ol fraction (%yield = 13.3%) and 22 g of the solid residue (%yield = 73.3%), all of which were insoluble in water.

Quantification of the proanthocyanidins and phenolic contents of HU and Hu_b: Based on the various methods adopted in the determination of the phenolic contents in HU and HU_b, the linear regression analysis of calibration plot gave a correlation coefficient (R^2) of 0.9218 for catechin, 0.9948 for rutin, 0.9814 for rutin used as standards in the determination of proanthocyanidins, flavonoids and total phenol, respectively. The proanthocyanidin contents of HU and HU_b extract were estimated to be 38.90±1.67 and 53.67±1.12 mg g^{-1} of dry extract, respectively while the total flavonoidal contents of HU and HU_b were estimated to be 0.50±0.03 and 11.78±1.47 mg g^{-1} of dry extract, respectively (Table 1). Also, the total phenolic contents in HU and HU_b were estimated to be 39.68±2.56 and 97.12±3.32 mg g^{-1} of dry extract (Table 1).

DPPH free radical scavenging activities of HU and its fractions: Using DPPH, the free radical scavenging activities of HU, HU_b and HU_{Af} and ascorbic acid (the reference drug) were observed to be dose dependent with HU_{Af} having the most significant ($p<0.05$) free radical scavenging activity (Fig. 1). Although, initially at 0.2-0.8 mg mL^{-1}, the free radical scavenging activity of

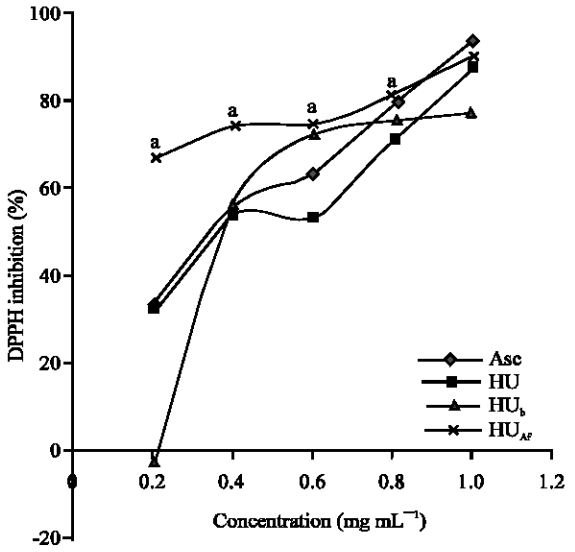

Fig. 1: DPPH free radicals scavenging activities of the crude aqueous seed extract (HU), butan-1-ol fraction (Hu_b) and alkaloid fraction (HU_{Af}) of *Hunteria umbellata* (K. Schum.) Hallier f. (Apocynaceae). a: Represents a significant increase at $p<0.05$ when compared to HU, HU_b and Asc values

Table 1: Quantification of polyphenol derivatives in HU and Hu_b

	Quantity (equivalent mg g^{-1} of dry extract)	
Polyphenol derivatives	HU	Hu_b
Total proanthocyanidins (tannins)[a]	38.90±1.67	53.67±1.12
Total flavonoids[b]	0.50±0.03	11.78±1.47
Total phenols[c]	39.68±2.56	97.12±3.32

[a]Expressed as mg catechin/g of dry extract. [b]Expressed as mg rutin/g of dry extract. [c]Expressed as mg rutin/g of dry extract, HU: Crude aqueous seed extract of *Hunteria umbellata*, HU_b: Butan-1-ol fraction of the crude aqueous seed extract of *Hunteria umbellata*

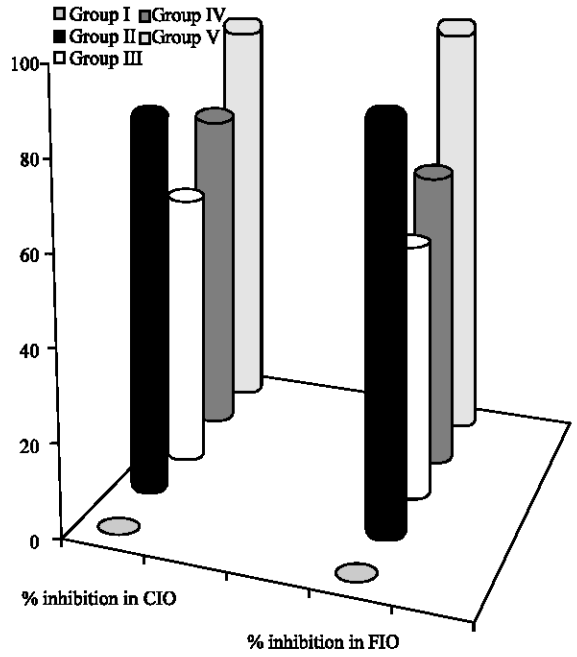

Fig. 2: Inhibitory effect of HU, Hu_b and HU_{Af} pre-treatment on in Carrageenan-Induced Oedema (CIO) and Formalin-Induced Oedema (FIO) in Wistar rat paws.

Table 2: Superoxide anion and nitric oxide scavenging activities of HU, HU_b and HU_{Af}

	Antioxidant activities (IC_{50} in $\mu g\ mL^{-1}$)	
Extract	Superoxide anion scavenging	Nitric oxide scavenging
HU	170.50±10.80	96.00±4.75
HU_b	112.00±2.50[a]	46.70±2.00[b]
HU_{Af}	85.00±0.45[c]	24.00±1.40[c]
Quercetin (standard)	83.00±0.50[c]	67.50±2.45[a]

[a-c]Represent significant decreases at $p<0.05$, $p<0.01$ and $p<0.001$, respectively, when compared to HU values, HU: Crude aqueous seed extract of *Hunteria umbellata*, HU_b: Butan-1-ol fraction of the crude aqueous seed extract of *Hunteria umbellata*, HU_{Af}: Alkaloid fraction of the crude aqueous seed extract of *Hunteria umbellata*

HU_{Af} was significantly ($p<0.05$) higher than either HU or ascorbic acid but higher concentration, the free radical scavenging activities of extracts and reference drug became comparable (Fig. 1).

Superoxide anion and nitric oxide scavenging activities of HU and its fractions: HU_b and HU_{Af} fractions significantly ($p<0.05$ and $p<0.001$) scavenged the superoxide generated by photo-reduction of riboflavin (Table 2). However, scavenging activity of HU_{Af} (IC_{50}: 85.00±0.45 $\mu g\ mL^{-1}$) was found to be greater than that of HU_b (IC_{50}: 112.0±2.50 $\mu g\ mL^{-1}$) but relatively comparable to relative to that of the standard drug, quercetin (IC_{50}: 83.00±0.50 $\mu g\ mL^{-1}$) (Table 2). Similarly, HU_b and HU_{Af} fractions significantly ($p<0.05$, $p<0.001$) inhibited the generation of nitric oxide from nitroprusside solution with the IC_{50} values of 46.70±2.00 and 24.00±1.40 $\mu g\ mL^{-1}$, respectively when compared to HU values (Table 2).

Anti-inflammatory activity of HU and its fractions: Oral pre-treatment with HU, Hu_b and HU_{Af} resulted in significant ($p<0.05$, $p<0.001$) inhibition of inflammation caused by carrageenan and formalin injections in the treated rats. However, the % inhibition caused by 50 mg kg^{-1} of HU, HU_b and HU_{Af} was more effective than that induced by the standard anti-inflammatory agent, aspirin (Fig. 2).

DISCUSSION

In the current study, the anti-inflammatory and antioxidant activities of HU and its fractions were evaluated using different standard methods. Carrageenan-induced rat paw oedema has been frequently used to screen natural products with anti-inflammatory potentials (Tapas *et al*., 2008). Inflammation induction with carrageenan involves the activation of platelet activation factor and release of pro-inflammatory mediators such as prostaglandins, kinins, tumor necrosis factor and nitric oxide (Tan-No *et al*., 2006). Carrageenan induces inflammation in three distinct major phases namely: the first phase which involves the release of histamine and serotonin, release of kinins in the second phase and the activation of cyclooxygenase-2 enzyme and ultimate release of prostaglandins in the third phase (Agbaje *et al*., 2008). Formalin has been reported to induce inflammation via similar mechanism as described for carrageenan (Joseph *et al*., 2009). However, non-steroidal anti-inflammatory drugs such as aspirin, indomethacin and diclofenac are known to mediate their anti-inflammatory action via inhibition of these phases of inflammatory response (Vane and Booting, 1987; Noguchi *et al*., 2005). It is, thus, possible that HU and its fractions mediate their anti-inflammatory action via inhibition of these inflammation phases as well. More importantly, results indicated that the anti-inflammatory activity of HU can be attributed to its alkaloid content whose anti-inflammatory effect was profoundly higher than that of the standard anti-inflammatory drug, aspirin. Another notable result of this study is the effective free radical scavenging and antioxidant effects of HU and its fractions, with HU_{Af} demonstrating the most significant free radical scavenging and antioxidant activities. Literature has shown that standard antioxidant drugs such as ascorbic acid and quercetin mediate their free

radical scavenging activities through proton donating mechanism (Wolfe *et al*., 2003; Adedapo *et al*., 2008). Thus, the results of this study suggest that the free radical scavenging and antioxidant activities of HU and its fractions may be mediated through this mechanism. Bearing in mind the strong association between inflammation and generation of ROS (Schreckinger *et al*., 2010), it is plausible that the anti-inflammatory activity of HU_{Af} is partly mediated via free radical scavenging and antioxidant mechanisms. Again, HU_{Af} may also account for the antioxidant activity of HU. Alkaloids and polyphenols have been reported to exhibit significant inhibitory activities on nitric oxide implicated in physiological and pathological process as chronic inflammation (Joseph *et al*., 2009). The presence of alkaloids in abundance (Adegoke and Alo, 1986) may account for the antioxidant activity of HU.

In conclusion, results of this study clearly indicate that the anti-inflammatory activity of HU could be attributed to its alkaloid content which was mediated via free radical scavenging and antioxidant mechanisms.

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