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Original Research Article

Inhibition and reversal of formaldehyde-induced inflammation in rats by active compounds isolated from leaf extract of Chikadoma plant (*Duranta repens*)

Sylvester C Ohadoma[1]*, Godwin C Akuodor[2], Lapah P Takem[1], Ekaette S Udoh[1], Arit Umoren[1], Ezechukwu I Nwokoma[3]

[1]Department of Pharmacology, Faculty of Basic Medical Sciences, College of Medical Sciences, University of Calabar, PMB 1115, Calabar, [2]Department of Pharmacology and Therapeutics, Faculty of Medicine, College of Health Sciences, Nnamdi Azikiwe University, Nnewi Campus, Anambra State, [3]RH/FP Department, Society for Family Health, Abuja, Nigeria

For correspondence: **Email: chodraf@yahoo.com; **Tel:** +234 8035081946*

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Abstract

Purpose: *To investigate the anti-inflammatory activity of the leaf extracts of Duranta repens.*
Methods: *Methanol, ethyl acetate, and hexane fractions from the methanol crude extract of Duranta repens leaves were obtained using gradient mixture elution in bioactivity-guided silica gel column chromatography. A mixture of spectrochemical, infrared (IR), nuclear magnetic resonance (NMR), and gas chromatographic/mass spectroscopic (GC/MS) investigations were used to clarify the structures of the identified active chemicals. A total of 50 Wistar rats randomized into 10 groups (n = 5) were given either 200 mg/kg of Hexane fraction (HFCP) or ethylacetate fraction (EAFCP) intraperitoneally. A subplantar injection of 0.1 mL 2.5 % formaldehyde solution was used to induce inflammation 1 h later, and this procedure was repeated on day three. After five days, the compounds were administered once daily, and then every other day for the next five days. Using a plethysmometer, the rat paw volume was measured every day following a 1 h drug delivery period. Diclofenac was used as the control.*
Results: *Compounds isolated belong to the following groups: flavonol glycoside, stigmast steroid and triterpenehydroxyl acid. The molecules identified by their structures are ursolic acid, stigmastene 3, 6-dione, and tetrahydroxy flavone-3α-rhamnoside, respectively. In rats, the combination of these compounds showed significant ($p < 0.05$) biological action against formaldehyde-induced inflammation, exceeding the respective percentage inhibition of individual compounds (62.60, 82.60, and 62.60 %; $p < 0.05$).*
Conclusion: *There is a significant anti-inflammatory activity of active compounds from the leaves of Chikadoma plant. The mechanism of this anti-inflammatory activity is through synergism. Clinical studies are necessary to advance the safety and toxicity profiles of these products.*

Keywords: *Anti-inflammation, Chikadoma plant, Yellow bush, Duranta repens, Active compounds*

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INTRODUCTION

Chikadoma plant (*Duranta repens*) known in English as yellow-bush has long been used to treat inflammation in Nigeria and West Africa [1]. It is a member of the Verbenaceae family [2]. Chikadoma plant is known for its vivid yellow blooms that are tinged with white and purple, and for being a bushy shrub that may reach a height of 1.8 m (6 ft) [1,3]. It is used in the

ethnomedicinal treatment of several illnesses, such as inflammation, pain, and microbiological infections. According to Wikimedia Common in 2020 and Wikiproject Plants/Archive 71, Chikadoma plant is named after Dr. Chika Ohadoma, a Nigerian pharmacologist who conducted considerable studies and popularized the ethnomedicinal potential of native flora in Nigeria [4,5].

Duranta repens possess anti-plasmodial properties, bactericidal, enzyme inhibition, and hypoglycemic effects due to the abundance of phytoconstituents [6-9]. Geographic variation has an impact on the chemical composition of plants [10]. For example, there are reports of first isolation of three C-alkylated flavonoids from *D. repens*, together with (+), 3, 13-clerodadiene-16,15-olid-18-oic acid, and (+)-hardwickiic acid [9]. Five recognized flavonoids were extracted from the chloromethane soluble fraction of *D. repens*, and two novel flavonoid glycosides were reported from the whole plant [6]. Additionally, it has been revealed that the methanol extract of leaves and stems of *D. repens* included a novel triterpenoid glycoside in addition to 14 other recognized chemicals, including one flavonoid, one phenyl ethanoid glycoside, four iridoides, and eight triterpenoids [10]. Traditional use of Chikadoma plant and its anti-inflammatory properties are well documented [1]. This study investigated the anti-inflammatory effect of the biologically active agents isolated from the leaves of Chikadoma plant.

EXPERIMENTAL

Material

Chikadoma plant, (*D. repens*), was collected in August 2022 from the surroundings of the University of Calabar in Nigeria. The plant was authenticated by Dr. Ebigwai Joseph of the Botany Department at the University of Calabar and allotted a voucher number (Bot/Herb/UCC/065). The leaves were allowed to air dry for 28 days at ambient temperature. The dried leaves were pulverized using an electric blender. The sample was cold macerated in methanol for 48 h and filtered. The extract was oven-dried after being concentrated at lower pressure using a rotatory evaporator. Standard techniques were used for phytochemical analysis of the extract [11].

Animals

Healthy adult Wistar rats (200 - 320 g) of both sexes were used for the anti-inflammation evaluation. The animals were made to acclimatize for 2 weeks under standard laboratory conditions (12 h light/dark cycle and unrestricted access to water and standard feed (Guinea Feeds Plc, Nigeria)). Ethical approval was obtained from the Faculty of Basic Medical Sciences, University of Calabar (approval no. UC/FBMS/21/033) and the study was conducted following the International Guidelines for Animal Studies [12].

Column chromatographic separation of crude methanol extract (CMECP)

Two-thirds of a glass column was filled with 200 g of activated silica gel (70 - 230 mesh). Thereafter 100 g of dry methanol extract was dissolved in a 1:1 v/v methanol: water mixture and transferred into the column. Hexane fraction (HFCP), ethylacetate fraction (EAFCP), and methanol fraction (MEFCP) were obtained by successively eluting the column with 1.5 L of hexane, 1.2 L of ethylacetate, and 1.0 L of methanol.

Isolation and purification of the active constituents from HFCP

Silica gel column chromatography was used to isolate a fraction (4.0 g) of the HFCP. The column was eluted using gradient mixes of hexane, ethylacetate and methanol. Thereafter, 20 mL aliquot was obtained and observed using UV spectral analysis, TLC, and phytochemical reactions. Fractions that were similar were bulked. The different fractions were studied for anti-inflammatory effects. The HFCP fraction (at the calibration levels on the glass column of 1-74 and 75 – 86) were eluted using hexane: ethylacetate (90:10 v/v), and 65 and 60 mg of the compounds were isolated respectively. Hexane: ethyl acetate (90: 10 v/v) was used to elute the produced product, which was then recrystallized in acetone before its purity was assessed using TLC-precoated with 0.25 mm silica gel 60 GF 254 (Merck). Using a combination of phytochemical study, melting point determination, ultraviolet (UV) spectroscopy, infrared (IR) spectroscopy, nuclear magnetic resonance (NMR), and gas chromatographic/mass spectroscopic (GC/MS) investigations, the structure of the isolated drug was clarified.

Tests for flavonoids

A concentrated sulfuric acid solution (1 mL) and 0.5 g magnesium were mixed with a fraction (5 mL) obtained from crude extract. Flavonoids are present when there is a pink or crimson tint that goes away after standing for 3 minutes.

Isolation and purification of the active constituents from EAFCP

A portion (4.0 g) of EAFCP was separated employing silica gel column chromatography. The gradient mixtures used for the column elution were hexane: ethylacetate (1:1 v/v) and hexane: ethylacetate: methanol (1:2:1 v/v). Thereafter, 20 mL of aliquot was taken, and TLC and UV spectral analysis were used for monitoring. The fractions that were similar were bulked. A 60 mg molecule was isolated from the EAFCP fraction (50 – 69 calibration levels on the glass column) that was eluted with ethylacetate: methanol (4:1 v/v) due to its bioactivity. This was purified by PTLC on a silica gel plate (0.5 mm) and developed using ethylacetate as the mobile phase. A combination of UV, FTIR, 1HNMR, and flavonoid tests were used to clarify the structure of the isolated chemical.

Formaldehyde-induced inflammatory test

This is a model of sub-acute/chronic inflammation. The approach outlined by Cui [13] was used as the inflammatory model but with some modifications. A total of 50 Wistar rats randomized into 10 groups (n = 5) were used in the study. On the first day, adult Wistar rats (n = 5; group = 10) were given either 200 mg/kg of HFCP or EAFCP intraperitoneally. A subplantar injection of 0.1 mL 2.5 % formaldehyde solution was used to induce inflammation 1 h later, and this procedure was repeated on day three. To make sure it could always be submerged to the same degree in the plethysmometer's measuring chamber, the right hind paw was painted with ink at the level of the lateral malleolus as a precaution. Rats were given distilled water or 10 % tween 80, diclofenac sodium (50 mg/kg, i.p.), and extract/fractions in the control, standard, and test groups, respectively.

Treatment

On day 1, a plethysmometer was used to measure the rat's right hind paw's basal paw volume (V_0). On day 1 (after V_0) and Day 3, the animals received an injection of 0.05 mL 2 % v/v formaldehyde in normal saline into the right paw's sub-plantar area. An hour following inflammation induction on the same day, the conventional medication (diclofenac) and extract/fractions were administered. After the first five days, the drug was administered once daily, and then every other day for the next five days. Using a plethysmometer, the rat paw volume was measured every day following a 1 h drug delivery period. According to Sanusi *et al* [14], V_0 represents the paw volume before the induction of inflammation (I) and V_f represents the paw volume. Following induction of inflammation, the delta volume (Eq 1) was used to assess the degree of swelling (edema):

$$I = V_f - V_0 \quad \ldots\ldots\ldots\ldots\ldots\ldots \quad (1)$$

where I is edema (inflammation) at a given time. Also, the anti-inflammatory effect of the extract/fractions usually, expressed in percentage (A) was calculated using Eq 2 [14].

$$A\ (\%) = (1 - I_t/I_c)100 \quad \ldots\ldots\ldots\ldots\ldots\ldots \quad (2)$$

where I_t is the mean inflammation (edema) value at a given time in treatment, while I_c is that of control.

Statistical analysis

Data was analyzed using GraphPad Prism Version 8.0.1 (GraphPad Software, Inc., U.S.A). Dunnett's multiple comparison test was used to compare the data following analysis with one-way or two-way analysis of variance (ANOVA). $P < 0.05$ was considered statistically significant.

RESULTS

Isolation and purification of the active constituents from EAFCP

Chromatography of the ethyl acetate fraction over silica gel yielded two major fractions. A major fraction (50 - 69 calibration levels on the glass column) which was of interest was eluted with a gradient mixture of ethyl acetate: methanol (4:1 v/v). The isolated fraction: yellow semisolid (60 mg), NMR (CD3OD, 200 MHz):δ 6.20(1Hd, J=2.2Hz, H-6) δ 6.37(1H d, J=2.2Hz, H-8) δ7.34(1Hd, J=2.2Hz, H-21), δ 7.31(1Hdd, Jab=8Hz, Jac=2.2Hz, H-41), δ 6.93(1H dd, J=2.2H, J=8.8Hz, H-5"), δ 7.76(1H, d, J=8.8Hz, H-61), δ 5.34(1Hd, J=1.4, H-111), δ 8.5(1H dd, H-2"),δ 4.2(1H dd, H-3"), δ 3.7(1H dd, H-4"), δ 3.30(1H m, H-5"), δ0.93(1H d, J= 5.8Hz, Me-6"), UV $\lambda_{EtoHmax}$ nm:257, 302sh, 352 IR VkBrmaxcm-1; 3500- 3200(OH), 2900, 2800, 1650 (c=O) 1600 (aromatic€=c), 1280, 1150, 750,730 (aromatic). This flavonol glycoside was identified as tetrahydroxyflavone-3α-rhamnoside compared with data (Figure 1).

Isolation and purification of the active constituents from HFCP

Two main fractions were obtained by chromatography of the hexane fraction on silica gel. The first fraction (1-74 calibration levels on

the glass column) of interest was eluted using a gradient mixture of hexane: ethyl acetate (90: 10 v/v), and it was crystallized as a white amorphous solid (65 mg). m.p:1380, 1HNMR (CDCl3, 200 MHZ): δ0.87 (3 Ht, J=5.69, H-23) δ0.72 (3 H-s, Me-18), δ0.95 (3 Hd, Me-27), δ1.00 (3 Hd, Me-26), δ1.18 (3 Hd, Me-21), δ2.10 (1 H, S, H-5), 13CNMR (CDCl3, 50 MHZ): δ99.4 (C-23 and C-24) δ206.8 (C-3 and C-6), and UV λCHCl3max nm (ε): 242 (31.8), 272 (30.6). IR VkBrmax Cm-1: 1700, 1429, 1369, 1150, 815, 820, 2950-2849. Stigmastene 3, 6-dion was determined to be the resultant stigmast steroid.

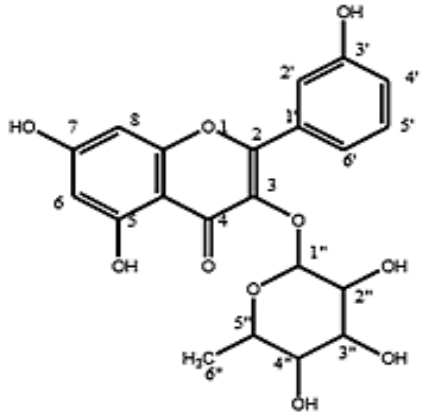

Figure 1: Structure of tetrahydroxylflavone-31α-rhamnoside

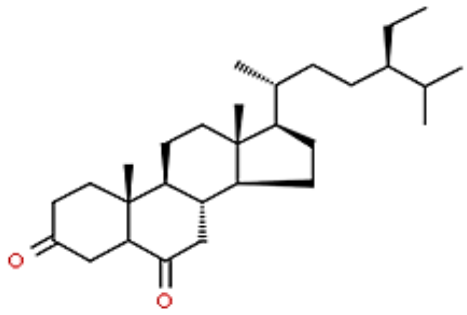

Figure 2: Structure of stigmastene 3, 6-dione isolated from Chikadoma plant

The subsequent fraction (75–86 calibration levels on the glass column), which is of importance in this study, was crystallized as a white crystalline solid (60 mg), m.p (202 °C), after being eluted using the same gradient mixture. Maximum wavelengths of UV (λ) CHCl3 (ε): 227.2 (1.321), 362.6 (0.07), 493.4 (0.034), and 3267 (OH). VkBrmaxCm1 in IR: 33002600 (wide, OH), 1678 (C=O), 1287, 1266, 1248, 1231, 1179, 1024, and 922,1HNMR (CDCl3, 200 MHZ): Ω 5.15 (1H, bs), 4.49 (1H,t, J=7.4(HZ), 2.01 (3Hs-OCOCH3), 1.60(3Hs), P.96(6Hs), 0.83 9(Hs), and 0.76 (3Hs). It was determined that this triterpene hydroxyl acid was ursolic acid (Figure 3).

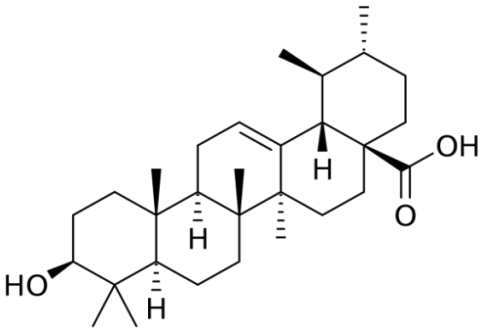

Figure 3: Structure of ursolic acid isolated from Chikadoma plant

Formaldehyde-induced inflammation

While all three of the active compounds from leaf extracts of Chikadoma plant significantly reduced paw edema when compared to negative controls, the simultaneous administration of the three compounds namely: ursolic acid (62.60 %), stigmastene 3, 6-dione (82.60 %) and tetrahydroxy flavone-3α-rhamnoside (62.60 %) showed significant anti-inflammatory effect ($p < 0.01$) on rat edema caused by formaldehyde. Thus, the percentage of inhibition following combined administration (88.51 %) was greater compared to individual compounds.

DISCUSSION

The primary active components in Chikadoma leaves that have anti-inflammatory effects on test animals include ursolic acid, stigmastene 3, 6-dione, and tetrahydroxyl flavone-3α-rhamnoside which is in tandem with previous studies [1,15]. The active ingredient, tetrahydroxyflavone-3α-rhamnoside, was extracted as a yellowish semisolid substance. The flavonoid nucleus ring B was identified by ^{1}H-NMR as having four aromatic protons that resonated at δ 7.34, 7.31, 6.93, and 7.76. Flavonoid nucleus ring A was assigned to two meta-linked (J = 2.2) aromatic protons that resonate at δ 6.2 and 6.37. No signal was detected for the proton in ring C, H-3 (δ 6.5 and 7.1). It implies that rather than flavones, the chemical is probably a flavonol. The sugar is most likely a rhamnose based on the chemical shift (δ 5.54 from the usual range 4.96-5.36) and the coupling constant 1.4 of the aromatic proton. A large peak was seen in the FT-IR spectra at 3500-3200 cm-1 (OH group), 1650 (C=O), and 1600 (C=C, aromatic). Typically, an up-field shift of H-21 and H-61 signals was caused by rhamnose or single sugar [16].

Table 1 A: Effect of the active fractions from leaf extract of Chikadoma plant on formaldehyde-induced rat paw edema

Group	Medication	Day 1	Day 2	Day 3	Day 4	Day 5
I	10% Tween 80 (0.4 mL)	0.482±0.026	0.558±0.023	0.627±0.025	0.832±0.017	0.855±0.015
II	Distilled water	0.515±0.031	0.615±0.030	0.640±0.019	0.812±0.024	0.827±0.021
III	Diclofenac sodium (50 mg)	0.363±0.027 (24.58)	0.308±0.032 (44.78)	0.267±0.023 (57.44)	0.643±0.025 (22.65)	0.563±0.026 (34.12)
IV	EAFCP (200) Flavonol glycoside	0.387±0.030 (19.72)	0.288±0.028 (48.36)	0.222±0.026 (64.62)	0.578±0.034 (30.47)	0.492±0.033 (42.49)
V	HFCP (200) Stigmast steroid	0.408±0.045 (15.24)	0.258±0.028 (53.73)	0.153±0.025 (75.54)	0.492±0.026 (40.88)	0.402±0.046 (53.02)
V1	HFCP (200) Triterpene hydroxyl acid	0.468±0.029 (23.85)	0.288±0.026 (48.36)	0.492±0.033 (42.49)	0.406±0.026 (41.76)	0.492±0.033 (42.49)
VII	EAFCP+HEFCP	0.423±0.023^{f} (17.81)	0.468±0.029^{f} (23.85)	0.388±0.034^{f} (39.33)	0.627±0.037^{f} (22.79)	0.567±0.033^{f} (31.45)
VIII	EAFCP+HEFCP	0.468±0.029 (23.85)	0.578±0.034 (30.47)	0.267±0.023 (57.44)	0.402±0.046 (53.86)	0.262±0.028 (53.13)
IX	HEF_1+HEF_2	0.383±0.029 (20.43)	0.262±0.028 (53.13)	0.192±0.031 (69.41)	0.600±0.045 (27.86)	0.580±0.047 (32.16)
X	EAF_1+HEF_1+HEF_2	0.393±0.025^{a} (18.35)	0.235±0.031^{a} (57.91)	0.138±0.022^{a} (77.93)	0.447±0.027^{a} (46.29)	0.333±0.026^{a} (61.02)

Table 1 B: Mean increase in paw volume (mL) ± SEM and percentage inhibition (A; %)

Group	Medication	Day 6	Day 7	Day 8	Day 9	Day 10
I	10% Tween 80 (0.4 ml)	0.797±0.022	0.715±0.027	0.610±0.039	0.522±0.034	0.450±0.035
II	Distilled water	0.758±0.020	0.698±0.016	0.600±0.017	0.553±0.023	0.492±0.031
III	Diclofenac sodium (50 mg)	0.498±0.026 (37.54)	0.442±0.022 (38.22)	0.370±0.027 (39.34)	0.327±0.027 (37.38)	0.262±0.024 (41.84)
IV	EAFCP (200) Flavonol glycoside	0.408±0.036 (48.75)	0.330±0.028 (53.85)	0.265±0.025 (56.56)	0.220±0.022 (57.83)	0.168±0.017 (62.60)
V	$HFCP_1$ (200) Stigmast steroid	0.315±0.037 (60.46)	0.227±0.032 (68.29)	0.172±0.027 (71.85)	0.122±0.020 (76.67)	0.078±0.015 (82.60)
V1	$HFCP_2$ (200) Triterpene hydroxyl acid	0.406±0.026 (41.76)	0.220±0.022 (57.83)	0.227±0.021 (59.03)	0.20±0.036 (55.18)	0.0168±0.017 (62.60)
VII	EAFCP+HEFCP	0.473±0.023^{f} (37.58)	0.406±0.026^{f} (41.76)	0.286±0.020^{f} (52.22)	0.227±0.021^{f} (59.03)	0.150±0.015^{f} (69.49)
VIII	EAFCP+HEFCP	0.265±0.025 (56.56)	0.330±0.028 (53.85)	0.408±0.036 (48.75)	0.353±0.039 (42.08)	0.262±0.024 (41.84)
IX	$HFCP_1$+$HFCP_2$	0.505±0.048 (36.61)	0.432±0.049 (40.80)	0.353±0.039 (42.08)	0.273±0.036 (47.61)	0.201±0.036 (55.18)
X	EAFCP+$HFCP_1$+$HFCP_2$	0.243±0.027^{a} (69.46)*	0.185±0.017^{a} (74.13)*	0.132±0.0213^{a} (78.41)*	0.097±0.011^{a} (81.46)**	0.052±0.010^{a} (88.51)**

Values are expressed as mean ± SEM (n = 5). Values in the bracket show % inhibition of paw edema. *P < 0.05, **p < 0.01, ^{a}p < 0.05 vs 80 % tween 80, ^{f}p < 0.05 vs distilled water

The active molecule, stigmastene 3, 6-dione, crystallized as a white amorphous solid and tested positive for steroids with a melting point of 138 °C. The existence of the stigmast nucleus was enforced by the ethyl group in the NMR spectra 3H d. The structural classification of steroids is based on the number of carbons in the molecules. Steroids with 29 carbons are referred to be stigmast steroids. It was clear from the significant infrared absorption at Vmax 1700 that the chemical was ketonic. Signals at δ 99.4 (C-23 and C-24) and δ 206.8 (C-3 and C-6) in the 13C-NMR spectrum confirmed the existence of one olefenic group and two ketonic groups. The signal in the infrared band at 815 cm^{-1} also confirmed this. Physicochemical characteristics of the product match those of 3, 6-dione [17]. Stigmast steroids that were extracted from *Vernonia colorata* leaves also exhibited anti-inflammatory properties [18]. In acetone, ursolic acid crystallized as a white crystalline solid with a melting temperature of 202 °C tested positive for steroids. Ursolic acid has been shown to have anti-inflammatory qualities in addition to several other physiological effects [19].

CONCLUSION

The primary active chemicals against formaldehyde-induced inflammation in rats found in the leaf extract of Chikadoma plant (Duranta repens) are ursolic acid, stigmastene 3, 6-dione, and tetrahydroxyflavone-3α-rhamnoside. These compounds exhibit synergistic anti-inflammatory effects. However, clinical studies are necessary to advance the safety and toxicity profiles of these products.

DECLARATIONS

Acknowledgement/Funding

None.

Ethical approval

Ethical approval was obtained from the Faculty of Basic Medical Sciences, University of Calabar, Calabar, Nigeria (approval no. UC/FBMS/21/033).

Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Conflict of interest

No conflict of interest is associated with this work.

Contribution of authors

We declare that this work was done by the author(s) named in this article, and all liabilities pertaining to claims relating to the content of this article will be borne by the authors. Ohadoma SC conceived and designed the study. All authors carried out the work, literature search, analyzed and interpreted the result. All authors read and approved the final draft of the manuscript for publication.

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