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Flavonol Glycosides from the Aerial Parts of *Indigofera hirsuta* and Anti-inflammatory Activity of n-butanol Fraction

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Authors' contributions

This work was carried out in collaboration between all authors. Author AMM Designed the study and wrote the protocol. Author LSA performed phytochemical investigation. Authors LSA, AMM, MIS and BB performed interpretation of compounds. Author LSA wrote the first draft of the manuscript. Authors AMM, LSA and MIA managed the literature searches. Authors MGM and LSA performed anti-inflammatory activity. Author MGM performed statistical analysis. Authors AMM, MIA, MIS and MGM reviewed. All authors read and approved the final manuscript.

Research Article

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ABSTRACT

Aims: The study aimed to phytochemically investigate the n-butanol soluble fraction of *Indigofera hirsuta* aerial parts and to evaluate the anti-inflammatory activity of the fraction using laboratory animal models.
Study Design: Isolation and elucidation of the bioactive compounds and anti-inflammatory activity investigation on n-butanol soluble fraction.
Place and Duration of Study: Department of Pharmaceutical and Medicinal Chemistry, Ahmadu Bello University, Zaria - Nigeria. The study was completed between January-

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October, 2011.
Methodology: The compounds isolated were identified using different spectroscopic techniques. The n-butanol fraction was investigated for its effect on carrageenan-induced oedema in rat's experimental model.
Results: Two Flavonol glycosides were isolated; Kaempferol-3-O-β-D-glucopyranoside (T2) and Kaempferol-7-O-β-D-glucopyranoside (Q3).The fraction significantly (P = .05) inhibited the carrageenan-induced paw oedema at doses of 150 and 300 mg/kg tested. The percentage anti-inflammatory effect of the highest dose tested (300 mg/kg) at the peak hour was higher than that of ketoprofen (10 mg/kg), the standard anti-inflammatory agent.
Conclusion: The result of this research suggests that the n-butanol soluble fraction of *Indigofera hirsuta* aerial parts contains bioactive compounds with anti-inflammatory activity.

Keywords: Indigofera hirsuta; fabaceae; kaempferol glycosides; anti-inflammatory activity.

1. INTRODUCTION

Chronic inflammation is the major risk factor for various types of disease [1]. It has been estimated that infectious and inflammatory reactions are linked to 15–20% of all cancer deaths [2]. Inflammation can lead to the development of diseases such as chronic asthma, rheumatoid arthritis, osteoarthritis, multiple sclerosis and inflammatory bowel diseases [3]. These diseases are debilitating and becoming increasingly common in our aging society. The two main classes of drugs employed in the management of inflammatory disorders; corticosteroids and NSAIDs do not result in complete cure. Moreover, severe side effects including obesity, hypertension, osteoporosis and increased susceptibility to infections remain the major challenges of corticosteroid therapy while gastrointestinal ulcerations, bleeding and platelet dysfunction are some of the serious side effects of NSAID drugs [4]. Therefore, search for more effective and safer anti-inflammatory agents from plant is a worthy research endeavour [5].

Indigofera hirsuta Linn (Fabaceae) is an annual herb found mostly in highlands of Northern part of Nigeria and in Angola [6]. In Nigeria, the plant is used in ethno-medicine for the treatment of diabetes, leprosy, tuberculosis, infections, snake bite and in the management of malaria and inflammation of the eyelids [6]. Methanolic extract of the aerial parts of this plant was shown to possess anti-bacterial activity [7], analgesic and anti-inflammatory activity [8]. Isolation of Stigmasterol from n-Hexane fraction of the methanol extract of this plant was reported [8]. In this study, we report the isolation and structural elucidation of two flavonol glycosides for the first time from n-butanol fraction and evaluation of the anti-inflammatory activity of the n-butanol fraction of *Indigofera hirsuta*.

2. MATERIALS AND METHODS

2.1 Phytochemical Investigation

2.1.1 Collection and Identification of Plant materials

The whole plant of *Indigofera hirsuta* (Fabaceae) was collected in the month of September, 2010 from Basawa village, Zaria, Kaduna state, Nigeria. The sample was identified by U.S.

Gallah of the herbarium section, Department of Biological Sciences, Ahmadu Bello University, Zaria-Nigeria, where, a deposited voucher specimen (No.390) was compared.

2.1.2 Extraction and isolation

The air dried aerial parts (800g) were exhaustively extracted with methanol (2.5L x 2) using Soxhlet apparatus for 48hrs. The crude methanol extract was concentrated to dryness using rota vapour and the yield obtained was 100g. It was then suspended in distilled water and filtered. The filtrate was successively partitioned into ethyl acetate, n-butanol and aqueous portions to afford EtOAc (4.7 g), n-BuOH (14.5 g) and H_2O (14 g) fractions respectively. The n-butanol soluble fraction (4g) was subjected to gel filtration over sephadex LH-20 column chromatography and eluted with methanol. A total of 52 fractions (5 ml each) were collected and pooled into eleven (11) major fractions (A – K) based on their TLC profiles, EtOAc-$CHCl_3$-MeOH-H_2O (15:8:4:1) solvent system. Repeated gel filtration of fraction H followed by preparative thin-layer chromatography led to the isolation of two compounds coded T2 [(9.7 mg, TLC R_f 0.62, EtOAc-MeOH-H_2O (100:16.5:13.5)] and Q3 [(7.2 mg, TLC R_f 0.71, EtOAc-MeOH-H_2O (100:16.5:13.5)]. All compounds were identified by a combination of spectroscopic methods and comparison with the literature data.

2.1.3 General experimental procedures

UV spectra were recorded on a Shimadzu UV-2500pc Spectrophotometer, IR spectra were run on a Shimadzu FTIR-8400S spectrophotometer, ^1H and ^{13}C 1D and 2D NMR spectra were recorded on a Bruker AVANCE III spectrometer operating at 400 MHz (^1H) and 100 MHz (^{13}C) using TMS as the internal standard with CD_3OD as solvents. Chemical shifts are reported in δ units and coupling constants (J) in Hz. Sephadex LH-20 (Pharmacia) was used for column chromatography. Thin-layer chromatography (TLC) was performed on a silica gel precoated glass plates (60 F_{254}, 20 x 20 and 0.30 mm thickness). Spots were visualized under UV lamp (254 and 365 nm), sprayed with Gibb's reagent/10% H_2SO_4 followed by exposure to ammonia solution and heating at a temperature of 110^0c for 5 minutes. All the solvent used were of analytical grade.

2.2 Anti-inflammatory Activity

2.2.1 Animals

Wistar rats (160-198 g) of both sexes were used. The animals were purchased from the animal House facility of the Department of Pharmacology and Therapeutics, Ahmadu Bello University, Zaria, Nigeria. They were kept in standard animal cages at room temperature and provided with standard laboratory diet and water *ad libitum*. The studies were conducted in accordance with the "Principles of laboratory animal care" [9].

2.2.2 Drugs and dosage

Ketoprofen injection (Lek, Slovenia; 10mg/kg) was used as standard drug, 1% solution of Carrageenan (sigma; 0.1cm^3/animal), n-butanol soluble fraction of the Methanol extract of *I. hirsuta* aerial parts (75, 150, and 300 mg/kg) and vehicle (Normal saline; DANA, Nigeria; equivalent volume administered with extract). All test solutions were administered intraperitoneally.

2.2.3 Acute toxicity study

The median Lethal dose (LD_{50}) of n-butanol fraction was determined using rats by [10] method, and was carried out in two phases. In the first phase, three groups of three rats each were administered with extract at doses of 10, 100 and 1000 mg/kg body weight intraperitoneally. They were observed for signs and symptoms of toxicity and death for 24h. In the second phase, four groups each consisting one rat were treated based on the result of the first phase. The 1st, 2^{nd,} 3rd and 4th groups were treated with the extract at doses of 200, 400, 800 and 1600 mg/kg, respectively. They were observed for signs and symptoms of toxicity and death for 24hr. The LD_{50} was calculated as the geometric mean of the lowest lethal dose that caused death and the highest non-lethal dose for which the animal survived.

2.2.4 Carrageenan-induced paw oedema

The test was conducted according to the [11] method. Thirty rats were divided into 5 groups, each consisting of six animals. Group 1(Negative control) received 1ml/kg saline. Group 2, 3 and 4 received fraction at doses of 75, 150 and 300 mg/kg respectively, while group 5 received positive control (ketoprofen; 10 mg/kg body weight). One hour later, 0.1ml of freshly prepared carrageenan suspension (1% w/v in 0.9% Normal saline) was injected into the sub plantar region of the left hind paw of each rat. The paw diameter was measured immediately before carrageenan injection and 1, 2, 3, 4 h after carrageenan injection using Vanier calliper.

2.3 Statistical Analysis

It was conducted using SPSS statistical package 17.0. Data were expressed as mean ± SEM. The mean values of the control group were compared with the mean values of the treated groups using one way ANOVA followed by Post hoc Dunnet's t-test for multiple comparison. Results were considered statistically significant at $P < 0.05$.

3. RESULTS AND DISCUSSION

Compound T2 was obtained as light yellow solid which reacted positively for flavonoids using shinoda and Ferric chloride reagents [12]. The UV spectrum (MeOH) showed absorption bands λ maxima at 346nm and 267nm characteristic of Kaempferol nucleus [13]. The IR spectrum showed a strong absorption bands at 3350 cm^{-1} (OH), 1637 cm^{-1} (C = O), 1592 and 1400 cm^{-1} (C = C aromatic functions), a good correlation to those of Kaempferol derivatives [14,15,16].

The ^{1}H-NMR spectrum (Table 1) displayed signals for two meta-coupled protons on a tetra-substituted benzene assigned to ring A at δ 5.93 (1H, d, J=1.9 Hz, H-6) and δ 6.11 (1H, d, J=1.9 Hz, H-8); 1′,4′-di-substituted benzene ring comprised of ortho-coupled AB system signals at δ 7.82 (2H, d, J=8.8 Hz) and δ 6.66 (2H, d, J=8.8 Hz) assignable to H-2′/H-6′ and H-3′/ H-5′ respectively on ring B, characteristic of a kaempferol nucleus [13,17,18].

The ^{13}C-NMR spectrum and the DEPT experiments indicated the presence of 21 carbon signals, 9 of them are quaternary carbons, and 7 were oxygenated, including the downfield signal at 179.5 due to a carbonyl (CO) group and one -CH_2 group. The ^{1}H signals around δ (3.46-2.97) corresponding to ^{13}C signals around δ (79-63) suggest the presence of one

sugar unit. Signal at δ 3.46 (dd, J = 2.2, 2.2 Hz) assigned to CH_2 of (H-6″) revealed the presence of glucosyl sugar moiety [13].

The hetronuclear correlation experiments (HSQC) established the attachment of glucose anomeric ^{1}H at δ 5.0 with its anomeric carbon at δ 104.8. The large coupling constant observed on the glucose anomeric proton (d, J=7.2) due to diaxial coupling with H-2″ confirmed the presence of β-glucosyl moiety [14].

In the HMBC spectrum, a cross-peak observed between δ 5.0 (H-1″) and 104.8 (C-3) established the connection of Kaempferol aglycone and β-glucosyl moiety. Thus, compound T2 was elucidated as Kaempferol-3-O-β-D-glucopyranoside (Fig. 1) through the comparison of several physical and spectroscopic data with those of literature [16,17,18].

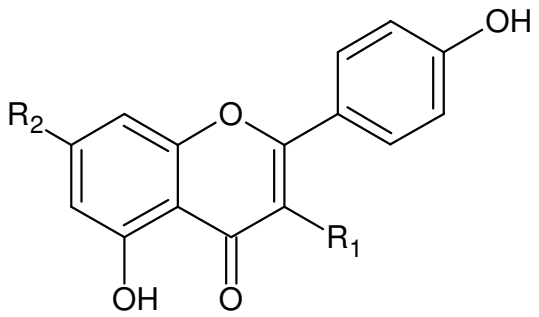
Table 1. ^{1}H and ^{13}C-NMR spectral data for compounds T2 and Q3 (CD_3OD), δ (ppm), J (Hz)

Position	Compound T2		Compound Q3	
	δ ^{13}C	δ ^{1}H	δ ^{13}C	δ ^{1}H
2	159.0	-	159.0	-
3	135.4	-	136.1	-
4	179.5	-	179.5	-
5	163.0	-	163.1	-
6	99.8	5.93 (d, 1.9)	102.1	6.40 (d, 2.0)
7	165.7	-	169.0	-
8	95.5	6.11 (d, 1.9)	97.0	6.61 (d, 2.0)
9	159.0	-	159.0	-
10	105.0	-	105.3	-
1′	122.0	-	122.8	-
2′, 6′	132.0	7.82 (d, 8.8)	132.3	8.07 (dd, 2.0, 8.9)
3′, 5′	116.0	6.66 (d, 8.8)	116.1	6.91 (dd, 2.0, 8.9)
4′	162.0	-	162.0	-
1″	104.8	5.00 (d, 7.2)	104.1	5.40 (d, 7.6)
2″	76.0	3.24 (s)	75.7	3.44 (t, 5.9, 7.0)
3″	79.0	3.20 (t, 6.68,7.72)	78.4	3.21 (m)
4″	72.0	3.16 (s)	72.0	3.48 (s)
5″	78.0	2.97 (m)	78.1	3.40 (s)
6″	63.0	3.46 (dd, 2.2, 2.2)	62.6	3.72 (dd, 2.2, 2.3)
		3.31 (dd, 5.4, 5.4)		3.56 (dd, 5.5, 5.5)

Compound Q3 was isolated as pale yellow solid and reacted positively for flavonoids using shinoda and Ferric chloride reagents [12]. The ^{1}H chemical shifts for ring B at δ 8.07 (dd, J=2.0, 8.9 Hz, H-2′/6′) and at δ 6.91 (dd, J=2.0, 8.9Hz, H-3′/5′) were typical of kaempferol.

The ^{1}H and ^{13}C-NMR spectral data (Table 1) indicated that Q3 is fundamentally similar structure to that of T2 except for the position of attachment of sugar moiety to the kaempferol aglycone. However, the down field resonance of C-7 in ring A suggested that the glucose unit in Q3 is attached to C-7. The observed HMBC correlations between H-1″ and C-6 and C-8 were also proof of the above assertion. Furthermore, the chemical shifts values at δ 5.40 for anomeric (H-1″, J=7.4 Hz), δ 6.40 for H–6 and δ 6.61 for H–8 and their corresponding carbons shifts at δ 104.1 (C-1″), 102.1 (C-6) and δ 97.0 (C-8) further suggested the attachment of sugar unit at 7 position [13]. This firmly confirmed the structure

of Q3 as Kaempferol-7-O-β-D-glucopyranoside through the comparison of ^{1}H-NMR and ^{13}C-NMR spectral data with those of literature data [19].



T2 R_1 = β-D-Glc; R_2 = OH
Q3 R_1 = OH; R_2 = β-D-Glc

Fig. 1. Structures of compounds T2 and Q3 isolated from n-butanol fraction of *Indigofera hirsuta*

3.1 Acute Toxicity Study

The lethal dose (LD_{50}) of the fraction was estimated to be 1264.91mg/kg in rats. This suggests that it is relatively toxic [10] but is relatively safe at the doses employed in the studies.

Table 2. Effect of n-butanol soluble fraction of the methanol extract of *indigofera hirsuta* and ketoprofen on carrageenan-induced paw oedema in rats

Treatment	Mean Paw diameter (cm) ± SEM, t(hr)				
	Dose(mg/kg)	1h	2h	3h	4h
Normal saline	1ml/kg	1.58 ± 0.42	2.30 ± 0.26	2.91 ± 0.24	2.10 ± 0.25
Extract	75	0.93 ± 0.18	1.84 ± 0.25	2.47 ± 0.33	1.71 ± 0.23
Extract	150	0.26 ± 0.06**	0.75 ± 0.18***	1.07 ± 0.09***	0.54 ± 0.12***
Extract	300	0.42 ± 0.11**	0.77 ± 0.08***	1.00 ± 0.12***	0.52 ± 0.09***
Ketoprofen	10	1.08 ± 0.13	1.37 ± 0.22	2.03 ± 0.16	1.46 ± 0.18

*P = .05; **P < .01; ***P < .001, compared to control, Dunnet's t-test, ANOVA (n=6)*

Table 3. Percentage inhibition expressed by n-butanol fraction of the methanolic extract of *Indigofera hirsuta* on carrageenan induced paw oedema in rat

	% Inhibition of oedema (time/h)				
Treatment	**[(Dose(mg/kg)]**	**1h**	**2h**	**3h**	**4h**
Extract	75	41.1	20	15.1	18.6
Extract	150	83.5	67.4	63.2	74.3
Extract	300	73.4	66.5	65.6	75.2
Ketoprofen	10	31.6	40.4	30.2	30.5

(n=6 for each treatment, experimental groups compared with control group.

The n-butanol fraction of *I. hirsuta* at doses of 150 and 300 mg/kg significantly (P = .05) inhibited the paw oedema over a period of 4 hrs (Table 2). The percentage anti-inflammatory effect of the highest dose tested (300 mg/kg) was higher than that of ketoprofen (10 mg/kg), the standard anti-inflammatory agent (Table 3). Carrageenan-induced inflammation is a commonly used acute inflammatory model. Carrageenan is the phlogistic agent of choice for evaluating anti-inflammatory potentials of compounds as it is not known to be antigenic and is devoid of apparent systemic effect [20]. The reproducibility of the model makes it a standard test in evaluating acute anti-inflammatory potential of compounds. The carrageenan-induced inflammation is bi-phasic, the first phase is due to the release of histamine, serotonin and kinins in the first hour; while the second phase is attributed to the release of prostaglandins and lysosomes in 2 to 3 hours [21]. The anti-inflammatory activity of the fraction might be in connection to the isolated flavonoids, since several flavonoids have been discovered to possess significant anti-inflammatory activity [22,23,24].

4. CONCLUSION

In conclusion, our results showed that the n-butanol soluble fraction of *I. hirsuta* contains flavonoid compounds with significant anti-inflammatory potential. This justifies the use of the plant parts for the management of inflammation and related inflammatory disorders.

CONSENT

Not applicable.

ETHICAL APPROVAL

All authors hereby declare that "Principles of laboratory animal care" (NIH publication No. 85-23, revised 1985) were followed, as well as specific national laws where applicable. All experiments have been examined and approved by the appropriate ethics committee.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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