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Research Article

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Anti-Inflammatory Constituents of *Randia hispida* K.Schum (Rubiaceae)

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ABSTRACT

Randia hispida K.Schum is a medicinal plant used by the Ijaws in the Niger delta region of Nigeria to treat inflammation, pyrexia and bacterial infection. In our continuing search for bioactive plant metabolites from Nigeria Medicinal plant as anti-inflammatory agents, the root bark of Randia hispida was investigated. The dichloromethane and 70% methanol extracts were screened for anti-inflammatory acitivity using xylene induced ear oedema in mice and egg albumin induced oedema in rats. The result revealed that dichloromethane extract at 486 mg/kg was the most active on both xylene induced oedema and egg albumin induced oedema with 66.7% inhibition which was comparable to dexamethasone 4mg/kg, while on egg albumin induced oedema at the same dose, the extract showed effect at the late phase of inflammation by inhibiting 80.3% inflammation compared to the standard drug used Acetyl salicylic acid (4mg/kg). Column chromatography of the dichloromethane extract using silica gel G and sephadex LH-20 led to the isolation of β-sitoistero(I) and ursolic acid (II). The structures of the isolated compounds were elucidated using NMR and MS and compared with literature data. This is the first report of these compounds from the plant. These compounds have been known to possess anti-inflammatory activity.

Keywords: *Randia hispida*; Anti-inflammation; β-sitosterol; Ursolic acid

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INTRODUCTION

Randia hispida is a small tree of about 10m tall found in farmland or forest. The Ibibios of Akwa Ibom state of Nigeria called it okukin ekpo,while the Ijaws of the Niger delta referred to it as Isack [1]. *Randia hispida* is used traditionally in the Niger delta region to treat pyrexia, inflammation and pain [1], while the decoction of the leaf is used to as enema against diarrhoea, dysentery and backache. Despite the ethnomedicinal usage of this plant by the local inhabitant of Niger delta area, there is no documentary evidence on the phytoconstituents of this plant. Some species of Randia are known to have ethnomedicinal application. Chemotaxonomy of this genus revealed that saponins,coumarins and triterpenoids have been isolated from these species [2,3]. In this present work, we report herein the anti-inflammatory activity of the extracts of this plant and the isolation of a steroid and a triterpene acid from the bioactive dichloromethane extract of the root of this plant.

EXPERIMENTAL SECTION

^{1}H and ^{13}C-NMR spectra were recorded on Pye-Unicam JEOL 500MHZ NMR spectrophotometer in $CDCl_3$ and DMSO with TMS as internal standard. IR was recorded on a Shimadzu 2140 IR spectrophotometer, while Mass spectroscopy was recorded on. Column chromatography was performed on silica gel G(200-400mesh,Silicycle),TLC was carried out on precoated silica gel G (0.2mm) aluminium backed (Silicycle), while gel filtration was performed using sephadex LH-20 (Sigma). Visualization was carried out by spraying with 10% sulphuric acid followed by warming.

Animals

The animals used in this study are: Swiss albino mice 23-35 g and wister rats (100-200 g) of either sex were bought from the animal house, Pharmacology department, University of Uyo and kept in the house under standard laboratory conditions as approved by the animals ethical committee of the University of Uyo. The animals were fed on standard diet and had free access to water. The animals were fasted for at least 12hour prior to the experiments.

Plant collection

The leaves of *Randia hispida* were collected from the wild at Otabi community in Oloibiri district of Ogbia local government area of Bayelsa State-Nigeria. The plant was authenticated by Dr Magaret Bassey, a plant taxonomist with the Department of Botany and Ecological science, University of Uyo where a voucher specimen UUH/033/15 was deposited in the herbarium.

Extraction

The dried pulverised stem bark was sequentially extracted at room temperature with 2x2.5 L each of n-hexane, dichloromethane and 70% methanol to exhaustion and the combined extracts were concentrated on a rotary evaporator to give a waxy mass of n-hexane 53.2 g (5.44%w/w),dichloromethane 26.4 g (2.7%w/w) and methanol 61.3 g (6.2%w/w).

Determination of acute toxicity

The acute toxicity test was carried out as described by [4] to establish the toxicity profile of the n-hexane,dichloromethane and methanol extracts. The LD_{50} were found to be 866mg/kg, 1620 mg/kg and 2371 mg/kg for n-hexane, dichloromethane and methanol respectively.

Xylene induced oedema

In this study, the method of [5] was adopted. Overnight fasted mice were divided into five groups (n=6). Group 1 was treated with distilled water (10 ml/kg), groups 2-4 received n-hexane extract 86.6 mg/kg, 173.2 mg/kg and 259.9 mg/kg, while group 5 received 4 mg/kg of dexamethasone. One hour later, one drop (0.03 ml) of xylene was applied to inner surface of the right ear to induce oedema. After 15 mins, the mice were killed under chloroform anaesthesia and both ears were cut off and sectioned circularly using a cork borer of diameter 7mm, the sections were weighed and % of inhibition of ear oedema was calculated relative to the left ear. The same procedure was repeated for dichloromethane extract 162 mg/kg, 324 mg/kg and 486 mg/kg, while for methanol extract the doses were 237.7 mg/kg, 474.3 mg/kg and 711.5 mg/kg.

Percentage inhibition was calculated as % inhibition = (ve-vt/vc)

Egg albumin induced inflammation

The methods of [6,7] were adopted. The overnight fasted rats 57-84 g were divided into five groups of six rats each. The first group received 10 ml/kg orally of normal saline, group 2 received Aspirin 100 mg/kg, while groups 3-5 received 86.6, 172.2 and 259.9 mg/kg of n-hexane extract, after 30mins,of administering the extract, inflammation was induced in the rat by injection of 0.1 ml/kg of egg albumin in the left hind paw of the animals. The volume and the linear circumference of the infected paw was measured before and 0.5,1,2,3,4 and 5 hours after administration of the phlogistic agent. The same procedure was for dichloromethane and methanol extracts.

Statistical analysis

The results were expressed as mean + SEM, statistical data was done using one way analysis of variance (ANOVA). The statistical analysis was done to determine the signifance of difference between the control groups and the treated groups. P values < 0.05 were considered to be significant.

Isolation

The bioactive dichloromethane extract (7 g) was packed in a column (3cmx50cm) with 50 g of silica gel and elution commenced gradiently with 100% n-hexane

Followed with n-hexane and ethylacetate mixtures at 95:5; 90:10; 80:20; 70:30; 60:40,50:50,30:70,10:90 and 100% ethylacetate. 50ml aliquots were collected and the progress of separation was monitored on TLC using the solvent systems: n-hexane: ethylacetate 5:1 and 2:1(solvent system 1 and 2 respectively). A total of 21 fractions were collected. Fractions 4-7 eluted with n-hexane: ethylacetate (90:10) gave 4 spots on TLC using solvent system 1 and were pooled together to give 0.15 g. This was purified over sephadex LH-20 eluting with 5% n-hexane in dichloromethane then 100%dichloromethane. 5mls aliquots, a total of 25 fractions were collected. Fractions 12-15 gave a single spot on TLC using solvent system 1 to give a white solid of compound I, (6 mg).

Fractions 14-16 from the column chromatography eluted with n-hexane: ethylacetate (50:50) which weighed 0.51 g was subjected to gel filteration over sephadex LH-20 and eluted with 100% dichloromethane. 10mls

aliquot were collected to give a total of 67 fractions. Separations were monitored on TLC using solvent system: n-hexane: ethylacetate (4:1). Fractions 33-53 were pooled together based on their TLC profile to give 0.1 g. This was subjected to column chromatography using silica gel G and eluted gradiently with n-hexane (100%) to n-hexane: ethylacetate (50:50). 10mls aliquot were collected. Fractions 10-18 which showed similar spots on TLC using solvent system 2 were pooled together to give compound II, a white amorphous solid (18 mg).

RESULT AND DISCUSSION

Compound 1, a white solid (6 mg)
IR (Nujol) cm^{-1}: 3423 (O-H Stretch), 1465(C=C) and 1063 (C-O)
^{I}H-NMR ($CDCl_3$) δ:ppm: 5.4(olefenic),3.5 (C-3 OH),0.7-1.5 (methyl and methylene protons)
EI-MS (Positive mode) m/z: 414 (M^+), 273(M^+-141), 255 (M^+-145-H_2O),43 (Isopropyl side chain)
Compound II, a white amorphous solid (15 mg)
^{I}H and ^{13}C-NMR spectra (Table 5)
ESI-MS (Positive mode): m/z 456 (M^+)
Acute toxicity of the two extracts gave the following LD_{50} of 1620 and 2371 mg/kg respectively, indicating that the extracts were moderately safe. The dichloromethane and methanol extracts showed marked inhibition of egg albumin induced oedema in rats, and this effect were comparable to the group that received the standard drug Aspirin. The oedema due to egg albumin inflammation is biphasic, and the early phase is mediated through the release of histamine,serotonins and kinins, while the late phase is mediated through the release of prostaglandins which is mediated by bradykinins and leucotriene produced by tissue macrophages. The anti-inflammatory activity shown by both dichloromethane and methanol extract was comparable to the standard drug. The results indicated that the two extracts acts on both early and late phase of inflammation. On xylene induced ear edema,both the dichloromethane and methanol extracts exhibited equal potency in inhibition of ear edema which were comparable to dexamethasone, the standard drug used (Table 4),this activity suggests the inhibition of phospholipase A2 which is involved in pathophysiology of inflammation due to xylene [8].

Table 1: Effect of dichloromethane extract on xylene induced ear oedema in mice

Treatment/Dose (mg/kg)	Weight of right ear (g)	Weight of left ear (g)	Increase in ear weight (g)	% Inhibition
Control Normal saline 10ml/kg	0.10±0.00	0.04±0.00	0.06±0.00	0
162.02	0.08±0.00	0.04±0.00	0.04±0.00[a]	33.33
324.04	0.07±0.00	0.04±0.00	0.03±0.00[a]	50
486.06	0.06±0.00	0.04±0.00	0.02±0.00[a]	66.67
Dexamethasone 4mg/kg	0.06±0.00	0.04±0.00	0.02±0.00[a]	66.67

Data are expressed as mean±SEM. Significant at [a]p‹0.001 when compared to control. n=6

Table 2: Effect of methanolic extract on xylene induced ear oedema in mice

Treatment/Dose (mg/kg)	Weight of right ear (g)	Weight of left ear (g)	Increase in ear weight (g)	% Inhibition
Control Normal saline 10ml/kg	0.10±0.00	0.04±0.00	0.06±0.00	
237.17	0.08±0.00	0.04±0.00	0.04±0.00[a]	33.33
474.34	0.06±0.00	0.03±0.00	0.03±0.00[a]	50
711.51	0.06±0.00	0.04±0.00	0.02±0.00[a]	66.67
Dexamethasone 4mg/kg	0.06±0.00	0.04±0.00	0.02±0.00[a]	66.67

Data are expressed as mean±SEM. Significant at [a]p‹0.001 when compared to control. n = 6

Table 3: Effect of dichloromethane extract on egg albumin induced paw oedema in Rats

	Time interval (hr)						
Treatment/dose(mg/kg)	**0**	**0.5**	**1**	**2**	**3**	**4**	**5**
Control Normal saline 10ml/kg	3.61±0.06	2.89±0.06	2.43±0.18	2.29±0.17	1.96±0.11	1.78±0.08	1.78±0.08
162	3.76±0.14	2.96±0.19	1.83±0.11[b]	1.64±0.11[a]	1.18±0.17[c]	0.95±0.15[b]	0.62±0.14[c]
324.04	3.63±0.04	3.19±0.13	2.55±0.12	2.09±0.11	1.65±0.14	1.22±0.10	0.73±0.10[c]
486.06	3.63±0.06	3.18±0.08	2.29±0.05	1.45±0.04[b]	1.29±0.04[b]	0.83±0.05[c]	0.35±0.04[c]
ASA 100	3.60±0.03	2.05±0.05	1.72±0.03[c]	1.49±0.05[c]	1.19±0.01[c]	1.05±0.01[a]	0.79±0.12[c]

Data are expressed as mean±SEM. Significant at [a]p‹0.05, [b]p‹0.01, [c]p‹0.001 compared to control. n=6

Table 4: Effect of methanolic extract on egg albumin induced paw edema in rats

	Time interval (hr)						
Treatment/dose(mg/kg)	**0**	**0.5**	**1**	**2**	**3**	**4**	**5**
Control Normal saline 10ml/kg	3.61±0.06	2.89±0.06	2.43±0.18	2.29±0.17	1.96±0.11	1.78±0.08	1.78±0.08
ME 237.17	3.44±0.07	3.16±0.05	2.79±0.08	2.39±0.11	2.19±0.05	1.99±0.09	1.47±0.07
474.34	3.39±0.08	2.83±0.19	2.76±0.16	2.19±0.19	1.76±0.18	1.33±0.18	1.15±0.18[b]
711.51	3.45±0.09	2.85±0.07	2.95±0.11	2.78±0.08	2.44±0.07[c]	2.14±0.09	1.89±0.11
ASA 100	3.60±0.03	2.05±0.05	1.72±0.03[c]	1.49±0.05[b]	1.19±0.01[c]	1.05±0.01	0.79±0.12[c]

Data are expressed as mean±SEM. Significant at [b]p‹0.01, [c]p‹0.001 when compared to control n=6,ME = methanol extract

Chromatographic separation of the active dichloromethane extract over silica gel and gel filtration on sephadex LH-20 led to the isolation of β-sitosterol (I) and ursolic acid (II).Compound I, a white solid gave a single spot on TLC with RF(0.33 and 0.48) using n-hexane:ethylacetate (9:1, 8:2). It gave a positive Lierbermann-Burchard test indicating that the compound was a sterol. Mass spectrum gave a molecular ion peak of m/z 414 corresponding to a molecular formula $C_{29}H_{50}O$. The IR spectrum showed the presence of OH group (3423cm-^{1}). The ^{1}H-NMR spectrum exhibited two signals at 0.65 and 1.00 ppm assigned to methyl groups at C-18 and C-19. The signals at δ 5.35 ppm in the ^{1}H-NMR spectrum is assigned to vinylic proton at C-5,while multiplets centered at δ =3.5 ppm is assigned to the hydroxymethine proton at C-3.The NMR and MS spectral data are in agreement with literature data of β- sitosterol [9,10]. Compound II was isolated as a white solid (12 mg), gave a positive Lierbermann-Burchard test suggestive of a steroid /triterpene.The mass spectrum gave a molecular ion peak of m/z 456 which translate to a molecular formula $C_{30}H_{48}O_3$. The ^{13}C-NMR spectra of compound II (Table 5), showed 30 signals consisting of of seven quaternary carbons, seven methine carbons, nine methylene carbons and seven methyl carbons which were deduced from the DEPT experiments. An olefenic proton at δ 5.12ppm in the ^{1}H-NMR spectrum alongside signals at 125.6 and 138.7ppm in the ^{13}C-NMR spectrum suggested it was ursane pentacyclic triterpene possessing a double bond between C-12 and C-13 carbons. Further signals at δ 178.7ppm in the ^{13}C-NMR spectrum together with IR absorption at 3420 cm^{-1} suggested the existence of a carboxyl group, compound II was found to be ursolic acid by comparison of the NMR spectral data with that reported in literature [11,12]. β-sitosterol have been known to possess anti-inflammatory activity [13]. Ursolic acid have been shown from previous reports to exhibit anti-inflammatory activities invivo, primarily observing reduced inflammation in mouse ear oedema models [14, 15]. The mechanism of anti-inflammatory activity of ursolic acid has been suggested to be mediated via the inhibition of histamine release, prostaglandins and leukotrienes production [16, 17].

Table 5: ^{1}H and ^{13}C-NMR Spectra of compound II in $CDCl_3$

Nr C H
1 38.7 -
2 27.5 -
3 77.3 3.01 (m)
4 38.7 -
5 55.2 -
6 18.5 -
7 33.2 -
8 40.1 -
9 47.5 -
10 36.8 -
11 40.1 -
12 125.6 5.12
13 138.7 -
14 42.1 -
15 30.7 -
16 24.3 -
17 47.5 -
18 52.8 -
19 38.9 -
20 39.7 -
21 30.7 -
22 37.0 1.19
23 23.7 1.25
24 16.6 0.98
25 15.7 0.77
26 17.4 1.08
27 23.3 1.14
28 178.7 -
29 21.5 0.93
30 17.5 0.91

CONCLUSION

From the results obtained, it can be concluded that the dichloromethane extract of *Randia hispida* exhibited anti-inflammatory activity based on the two models used, and the presence of β-sitosterol and ursolic acid, known anti-inflammatory agents could be responsible for the anti-inflammatory properties of this plant hence justifying the ethnomedicinal use of *Randia hispida* in relieving inflammation.

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