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Cyclooxygenase inhibition and antimycobacterial effects of extracts from Sudanese medicinal plants

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Abstract

Fifty one dichloromethane, ethyl acetate and ethanol extracts obtained from seven tree species used in Sudanese traditional medicine were screened for *in vitro* anti-inflammatory activity using COX-1 and COX-2 assays and for antimycobacterial activity using the broth micro-dilution methods against *Mycobacterium aurum* A+. In the cyclooxygenase assays, all ethyl acetate (leaf, bark, root) and ethanol root extracts of *Acacia seyal*, ethyl acetate twig extracts of *Capparis decidua*, dichloromethane bark extracts of *Combretum hartmannianum* and ethanol bark extracts of *Ziziphus spina-christi* showed inhibitory effect against prostaglandin synthesis by COX-2 ranging from 58 to 97% and weak (<50%) or no activity against COX-1 induced prostaglandin production. In the antimycobacterial assays, ethanol extracts of *A. seyal* (bark), *C. hartmannianum* (leaf, bark), *Kigelia africana* (bark) and *Z. spina-christi* (bark) inhibited growth of *M. aurum* A+ with MIC values ranging between 0.19 and 1.56 mg/ml. The *in vitro* anti-inflammatory activity observed in this study support the utilization of these plants in Sudanese traditional medicine as crude anti-inflammatory agents. The inhibition of COX-1 and COX-2 and the antimycobacterial effects of these plants have not been reported previously. Isolation of bioactive compounds from *A. seyal* and *Erythrina latissima* are in progress in our laboratory.

Keywords: Anti-inflammatory; Antimycobacterials; Medicinal plants; Traditional medicine

1. Introduction

The use of traditional medicine is expanding globally. It continues to be used not only for primary health care in developing countries, but also in countries where conventional medicine is predominant in the national health care system (World Health Organization, 2000). *In vitro* pharmacological investigations of traditionally used medicinal plants offers an incredible opportunity to explore and investigate a wide range of plant-based drugs as a potential source of novel biologically active agents and/or to validate claims made on their safety and efficacy (Gautam et al., 2007).

Inflammation is a succession of changes which occurs in living tissue when it is injured and/or is the reaction to injury of living microcirculation and related tissues (Punchard et al., 2004). Cyclooxygenase enzymes exist in two isoforms (COX-1 and COX-2), coded by distinct genes on different chromosomes. The two isoforms show about 50% homology and have similar catalytic activity, but are physiologically distinct. COX-1 is generally constitutive. It has housekeeping functions including gastric cytoprotection and platelet aggregation. In contrast, COX-2 is inducible in inflammatory cells. Though COX-2 can also be constitutive in some tissues, its expression and activity is largely responsive to adverse stimuli, such as inflammation (Pasinetti, 2001; Cronstein, 2002).

Tuberculosis continues to be an enormous global concern; it is spreading and infects millions of people annually (Janin, 2007). *Mycobacterium tuberculosis* infection has recently been increasing with a rising rate of coexisting human immunodeficiency virus (HIV). The search for new agents is needed due to the emergence of drug resistant strains of mycobacterials (Suksamrarn et al., 2003; Lee et al., 2006; Jiménez-Arellanes et al., 2007).

Ongoing research in our laboratory has focused on investigating the biological activities of some plants used in Sudanese

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traditional medicine. The reported uses for these Sudanese traditional plants are for the treatment of various ailments such as infectious diseases, central nervous disorders and ailments of an inflammatory nature (Iwu, 1993; Hutchings et al., 1996; Neuwinger, 1996; El Ghazali et al., 1997). *Acacia seyal* Del., *Erythrina latissima* E.Mey, *Capparis deciduas* (Forssk.) Edgew. and *Combretum hartmannianum* Schweinf. amongst others showed good antimicrobial and anti-cholinesterase activities during previous *in vitro* investigations, (Eldeen and Van Staden, 2007). In this report, anti-inflammatory and antimycobacterial activities of extracts obtained from some trees used in Sudanese traditional medicine are highlighted.

2. Materials and methods

2.1. Plant material

Plant materials (leaves and/or twigs, roots, bark) were collected from Shambat, Khartoum north-Sudan in December 2005. The plants were authenticated at the Department of Silviculture, Faculty of Forestry, University of Khartoum, Sudan and voucher specimens deposited in the Herbarium (Table 1). Botanical names, voucher specimen numbers and summary of traditional uses of the investigated plant species are recorded in Table 1.

The collected materials were dried in an oven at 50 °C for five days, powdered and extracted sequentially using dichloromethane, ethyl acetate and ethanol (10 mg/ml) by sonication for 1 h. The extracts were filtered using Whatman No.1 filter paper and dried under a fan at room temperature.

2.2. Anti-inflammatory activity of the plant extracts using the cyclooxygenase assays

2.2.1. Cyclooxygenase assays (COX-1 and COX-2)

Anti-inflammatory activity of the plant extracts was determined using both the COX-1 and COX-2 assays. The basic protocol is the same for both assays, allowing a comparison of the inhibitory effects of the extracts on the two enzymes. The COX-1 bioassay was performed according to the method described by Jäger et al. (1996). The COX-1 enzyme (isolated from ram seminal vesicles) (Sigma-Aldrich) was activated with co-factor solution and pre-incubated on ice for 5 min. Sixty µl of this enzyme/co-factor solution were added to 20 µl of test solution (2.5 µl of the plant extract +17.5 µl water) and pre-incubated for 5 min at room temperature. Twenty µl of [^{14}C] arachidonic acid were added to the test samples and incubated at 37 °C for 10 min. After incubation, the reaction was terminated by adding 10 µl 2 N HCl. Four µl of a 0.2 mg/ml carrier solution of unlabelled prostaglandins were added. Prostaglandins were separated from the unmetabolised arachidonic acid by silica gel column chromatography. The samples were loaded onto individual Pasteur pipettes packed with silica gel (particle size 0.063–0.200 mm, Merck). Four ml of hexane:dioxane: acetic acid (350:150:1 v/v) mixture were added, 1 ml at a time, to the column to elute the arachidonic acid. The prostaglandins were then eluted by the addition of 3 ml ethyl acetate:methanol (425:75 v/v) mixture and received in scintillation vials individually. Four ml scintillation solution were added to each vial containing the prostaglandins and radioactivity was measured using a Beckman L S 6000LL scintillation counter.

The COX-2 assay described by Noreen et al. (1998) with slight modifications (Zschocke and Van Staden, 2000) was followed. Human recombinant COX-2 containing a six histidine sequence near the N-terminus isolated from a Baculovirus over expression system in S*f* 21 cells was used (Sigma-Aldrich). The same protocol as for COX-1 was followed except that in the preparation of co-factor solution where 0.3 and 0.6 mg adrenaline was used with COX-1 and COX-2, respectively. In each test assay, four controls were run. Two were background in which the enzyme was inactivated with HCl before the addition of [^{14}C] arachidonic acid, and two were solvent blanks. Indomethacin was included in each test assay as a standard (5 µM for the COX-1 assay and 200 µM for the COX-2 assay). Both assays were performed in duplicate with double determinations for each sample per assay. Percentage inhibition by the tested compound was calculated by comparing the amount of radioactivity present in the sample to that in the solvent blank.

Table 1
Plant name/authority, family, voucher number, traditional uses and references of tree species used in Sudanese traditional medicine

Plant name	Family	Voucher	Traditional uses in Sudan and other African countries
Acacia seyal Del.	Mimosaceae	Ibra1	Arthritis rheumatism and rheumatic fever (El Ghazali et al., 1997).
Balanites aegyptiaca (L.) Del.	Balanitaceae	Ibra2	Venereal diseases, rheumatism, digestion problems, dysentery and bilharzias (Iwu, 1993; El Ghazali et al., 1997; Van Wyk et al., 1997).
Capparis decidua (Forssk.) Edgew.	Capparidaceae	Ibra3	Jaundice, rheumatic arthritis and to treat swells (El Ghazali et al., 1997).
Combretum hartmannianum Schweinf.	Combretaceae	Ibra4	Arthritis rheumatism, skin dryness, jaundice and bacterial diseases (Iwu,1993; Neuwinger, 1996; El Ghazali et al., 1997).
Erythrina latissima E.Mey.	Papilionaceae	Ibra5	Bronchial infections, coughs, wounds, abscesses, arthritis and throat inflammation (Iwu,1993; Hutchings et al., 1996; Van Wyk et al., 1997).
Kigelia africana (Lam.) Benth.	Bignoniaceae	Ibra6	Stomach problem, cough, dysentery and venereal diseases (Neuwinger, 1996; El Ghazali et al., 1997).
Ziziphus spina-christi (L.) Willd.	Rhamnaceae	Ibra7	Ulcers and gonorrhea, sore throat, chest pain, dysentery, venereal diseases and wounds (Neuwinger, 1996; El Ghazali et al., 1997; Dafni et al., 2005).

2.3. Antimycobacterial activity of the plant extracts using a strain of Mycobacterium aurum A+

2.3.1. Media and growth conditions

Middlebrook 7H10 agar base (ref 453982) and Middlebrook 7H9 broth base (ref 454012) were used. The supplement OADC (oleic acid + albumin + dextrose + catalase) (Remel, USA) was added (10%) to both agar and broth media. A stock culture of *Mycobacterium aurum* A+ was obtained from the Microbiology Laboratory, Division of Pharmacology, University of Cape Town. The bacterium was first isolated from human sputum in France in 1965 (Tsukamura, 1966). The organism (stock number CIP 104482) was used as indicated in the Standard Operating Procedures (SOP no. GL2005/01) for determination of minimum inhibitory concentration (MIC) of *M. aurum* as used in Microbiology Laboratory, Cape Town. Two (5 ml) supplemented 7H9 broths (Middlebrook 7H9 + 10% OADC) were inoculated by the stock bacterial culture and grown for 72 h at 37 °C. Gram and Ziel–Neelson stains were performed on these cultures to ensure culture purity. Twenty percent sterile glycerol was added to each culture and 500 µl aliquots were made into sterile 1.5 ml Eppendorf tubes. These stocks represent G0 stocks and were stored at −70 °C. A single stock was used to inoculate two further broth cultures. Further glycerol stocks were prepared from these cultures as described above and stored at −70 °C, representing G1 stocks. A single G1 stock was used to inoculate two supplemented Middlebrook 7H10 agar (7H10 + 10% OADC) plates and incubated at 37 °C for 4 days. From this culture a single colony was used to inoculate 5 ml supplemented 7H9 broth. This was grown at 37 °C for 72 h and used for the experiment.

2.3.2. Broth micro-dilution method

The broth microdilution method (Swenson et al., 1982) was used to determine the minimum inhibitory concentration (MIC) of *M. aurum* A+ of the investigated plant extracts using 96-well microtiter plates. The extracts were dissolved in water:ethanol (1:1 v/v). One hundred microliter of the supplemented 7H9 broth were added to each well using a multi-channel pipette. A further 100 µl of supplemented 7H9 broth were added to all the wells of the first column to serve as a medium control and blank. For the drug and solvent controls, as well as the test samples, 100 µl of the respective sample were added to the first well of the column. A multi-channel pipette was used to make a two fold serial dilution starting with a concentration of 25 mg/ml of the extracts and 62.5 µg/ml of the ciprofloxacin (standard drug control). The optical density (OD) of the 72 h liquid culture was adjusted to 0.125 at 550 nm. One hundred microliter of the diluted culture were added to every well of the microtitre plate excluding the wells of the first column that served as the medium control. The plates were loosely sealed in plastic bags to prevent them drying out and incubated at 37 °C for 72 h. After incubation, 40 µl of 0.4 mg/ml solution of *p*-iodonitrotetrazolim salt (INT) were added to each well of the plate. The plates were left sealed in plastic packets overnight at 37 °C. The lowest concentration containing no indication of red colour as a result of INT was deemed to be the MIC.

3. Results

3.1. Inhibition of cyclooxygenase enzyme activity

Inhibition of cyclooxygenase (COX-1 and COX-2) enzyme activity by the plant extracts was recorded as percentage inhibition of prostaglandin biosynthesis. Results expressed as mean ± S.D are shown in Table 2. A minimum inhibition of 50% is required for plant extracts to be considered active. All ethyl acetate (leaf, bark, root) and ethanol root extracts of *A. seyal*, ethyl acetate twig extracts of *C. decidua*, dichloromethane bark extract of *C. hartmannianum* and ethanol bark extracts of *Ziziphus spina-christi* showed good activity against COX-2 (inhibition (%) of prostaglandins ranging from 58 to 97%) and weak or no activity against COX-1 (inhibition (%) of prostaglandins <50%). Extracts from *Kigelia africana* (exceptions were leaf ethanol and bark dichloromethane extracts) showed only activity against COX-1. *E. latissima* (dichloromethane and ethanol bark and root extracts), *Z. spina-christi* (dichloromethane and ethyl acetate leaf and bark extracts), *A. seyal* (ethanol leaf and bark extracts), *Balanites aegyptiaca* (dichloromethane bark extract) possessed activity against both COX-1 and COX-2. With the exception of *K. africana* and *Z. spina-christi*, all ethyl acetate extracts showed no activity against COX-1.

3.2. Antimycobacterial activity of the tested plant extracts as detected against Mycobacterium aurum A+

A strain of the rapidly growing, non-pathogenic *M. aurum* A+ was used in this study due to the slow growth rate and the highly infectious nature of *M. tuberculosis*. Inhibition of *M. aurum* A+ growth is highly predictive of activity against *M. tuberculosis* (Chung et al., 1995).

The minimum inhibitory concentrations (MIC) of the plant extracts against *M. aurum* A+ as determined using the broth micro-dilution method are shown in Table 2. Ethanol extracts of *A. seyal* (bark), *C. hartmannianum* (leaf and bark), *K. africana* (bark) and *Z. spina-christi* (bark) inhibited growth of *M. aurum* with MIC values ranging from 0.19 and 1.5 mg/ml. Dichloromethane and ethyl acetate extracts showed weak activity against *M. aurum* in this study. Exceptions were *C. hartmannianum* (dichloromethane leaf extract) and *E. latissima* (dichloromethane root and bark and ethyl acetate bark extracts).

4. Discussion and conclusions

A. seyal is used traditionally in Sudan as a cure for colds, jaundice, headache and burns (Sahni, 1968). Women in Sudan use bark and wood of *A. seyal* and *Combretum* species for making a so-called smoke bath. Exposure to the smoke bath is believed to relieve rheumatic pains, smooth skin and achieve general body relaxation (Ogbazghi and Bein, 2006). Exposure to the smoke bath from wood of *A. seyal* is also used widely in Sudan (not documented) to accelerate recovery from an episiotomy cuts and/or tears in the cases of operative vaginal deliveries (Macleod and Murphy, In press), beside other

Table 2
Inhibition (%) of prostaglandin synthesis and minimum inhibitory concentration (MIC) values against *Mycobacterium aurum* A+ by plant extracts obtained from trees used in Sudanese traditional medicine

Plant species	Plant parts analyzed	COX-1 inhibition (%)			COX-2 inhibition (%)			MIC values (mg/ml)		
		Extracts tested (250 μg/ml)			Extracts tested (250 μg/ml)			Plant extract tested		
		Dichloro-methane	Ethyl acetate	Ethanol	Dichloro-methane	Ethyl acetate	Ethanol	Dichloro-methane	Ethyl acetate	Ethanol
Acacia seyal	Leaf	55±3.1	40±1.9	69±3.1	84±2.9	89±2.9	90±2.7	12.5	25	3.12
	Bark	74±3.0	12±1.7	68±5.5	39±3.1	96±4.6	96±1.3	25	25	0.78
	Root	96±2.8	46±2.6	45±5.2	67±2.6	94±3.8	97±5.5	12.5	12.5	3.12
Balanites aegyptiaca	Leaf	40±3.0	42±3.9	76±2.4	90±1.1	88±1.5	50±3.3	12.5	na	3.12
	Bark	77±5.1	09±3.6	91±1.7	72±4.4	76±4.9	52±1.3	12.5	na	12.5
	Root	97±4.0	07±3.0	98±1.5	28±2.3	48±3.8	48±4.9	25	12.5	12.5
Capparis decidua	Twigs	58±3.4	03±1.3	52±2.2	79±3.4	81±1.4	58±4.0	12.5	25	6.25
	Root	77±5.9	26±2.7	98±2.1	60±3.9	22±3.4	36±2.9	25	na	6.25
Combretum hartmannianum	Leaf	94±1.9	05±2.1	62±1.6	66±2.7	90±5.1	52±3.6	0.78	3.12	0.19
	Bark	10±2.1	19±2.9	57±2.6	90±1.5	34±3.8	41±2.9	12.5	25	1.56
	Root	48±1.2	34±3.1	42±3.1	20±4.0	29±2.1	39±2.4	3.12	na	12.5
Erythrina latissima	Bark	97±2.3	31±2.4	54±1.6	81±2.0	82±0.9	53±1.6	1.56	1.56	0.39
	Root	96±2.2	48±3.1	77±2.6	74±2.2	76±2.1	57±1.0	0.39	12.5	6.25
Kigelia africana	Leaf	72±2.9	91±3.5	42±4.4	22±5.2	16±2.9	19±4.3	6.25	12.5	6.25
	Bark	87±3.1	98±1.0	67±0.9	60±3.3	24±4.4	29±1.5	3.12	12.5	1.56
Ziziphus spina-christi	Leaf	83±3.4	96±2.8	04±2.2	84±2.5	84±4.1	51±1.0	12.5	12.5	6.25
	Bark	66±2.1	97±1.1	0.3± 1.1	79±1.9	88±1.2	58±2.4	25	6.25	0.39

Inhibition of prostaglandin and the MIC values were determined using the cyclooxygenase (COX-1 and COX-2) assays and the broth micro-dilution method respectively.
Inhibition obtained (%) is expressed as mean±S.D. Percentage Inhibition of prostaglandin synthesis by indomethacin (standard) was 80±1.9% for COX-1 and 69±2.4% for COX-2. na = not active at the highest concentration tested (25 mg/ml). Minimum inhibitory concentration (MIC) of ciprofloxacin (standard) against *M. aurum* in this study was 2 μg/ml.

cosmetic and medicinal beautification (Ogbazghi and Bein, 2006). Some of these symptoms are treated clinically by anti-inflammatory drugs (Lewis and Elvin-Lewis, 2003). Anti-inflammatory activity showed by these plants supports their traditional uses in Sudanese traditional medicine. Our findings also support the traditional uses and the reported anti-inflammatory activity of *C. decidua* (Ageel et al., 1986). Several chemical compounds were previously isolated from the investigated plant species. Capparisine, capparisinine and L-stachydrine and isocodonocarpine isolated from *C. deciduas* (Ageel et al., 1986), quercetin, quercetin-3-o-[β-xylosyl-(1-2)-α-rhamnoside]4′-o-α-rhamnoside were isolated from *Z. spina-christi* (Shahat et al., 2001). *E. latissima* was reported to produce erythrina-type alkaloids such as (+)erythraline, (+)-11-hydroxyerysodine, (+)-erythratidine and (+)-erysosalvine (Majinda et al., 2001). Norviburtinal, octacosanol, kigelin and stigmasterol amongst others occur in *K. africana* (Neuwinger, 1996). Inhibition of cyclooxygenase enzyme activity and/or antimycobacterial effects showed by the plant extracts in this study may be due to the presence of one or more of these chemical compounds. Tannin is a general descriptive name for a group of polymeric phenolic substances (Cowan, 1999). They affect highly purified enzyme-based targets due to their ability to bind strongly with proteins (Cowan, 1999). This may, in the case of anti-inflammatory testing, lead to the inhibition of prostaglandin synthesis through blocking the cyclooxygenase enzymes.

The mode of antimicrobial action of tannins may also be related to their ability to inactivate microbial adhesions, enzymes and cell envelope (Cowan, 1999). Tannins therefore, may be responsible, in part, for some of the anti-inflammatory and/or antimycobacterial activities showed by some of the plant extracts investigated in this study.

Inhibition of COX-1, as mentioned earlier, may lead to adverse effects on the gastrointestinal mucosa which may cause gastric ulceration and increase the risk of adverse cardiovascular events. However, some authors also reported that COX-2 is constitutively expressed in some tissues (Cronstein, 2002). This suggests that prostaglandins produced by COX-2 play an important role in the biological and/or physiological functions of such tissues (Cronstein, 2002). As some of the investigated plant extracts showed activity against COX-1 or both COX-1 and COX-2 in this study, the use of these plants as traditional anti-inflammation agents should be carefully monitored.

In a previous investigation, 75% of the currently investigated plant extracts showed antibacterial activity with MIC values less than/or around 1.5 mg/ml against *Bacillus subtilis*, *Staphylococcus aureus* (Gram-positive), *Escherichia coli* and *Klebsiella pneumoniae* (Gram-negative) (Eldeen and Van Staden, 2007). However, in this study only 19% of the same plant extracts showed activity against *M. aurum* A+ with MIC value less than/or around 1.5 mg/ml. Poor activity shown by some of the plant extracts may, in part, be due to a lack of uptake by *Mycobacterium* species due to the complex lipoglycan on the cell surface which provide a strong physical barrier to intracellular-acting agents (Oliva et al., 1998; Ballell et al., 2005). In contrast, some extracts (ethanol bark extract of *E. latissima* and leaf extract of *C. hartmannianum*) showed both antibacterial (Eldeen and Van Staden, 2007) and antimycobacterial activities in the present

study. This may be due to the presence of bioactive agents in these extracts that inhibit bacterial protein synthesis and could therefore be expected to exhibit antimicrobial activity against a range of bacteria including mycobacteria (Oliva et al., 1998). However, the mechanism of action of many of antimycobacterial drugs is poorly understood (Oliva et al., 1998; Janin, 2007).

Anti-inflammatory and antimycobacterial activity observed by some of the investigated plant extracts in this study indicate the presence of bioactive agents that warrant further investigation. Ongoing research in our laboratory is currently focused on the isolation and identification of bioactive compounds from *A. seyal*, *C. decidua*, *E. latissima* and *Z. spina-christi*.

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