

Full Length Research Paper

Anti-inflammatory activity and a new compound isolated from aerial parts of *Myrsine africana*

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A new compound was isolated from the ethyl acetate (EtOAc) fraction of the aerial parts of *Myrsine africana*. The compound was eluted with mobile phase of EtOAc and *n*-hexane at a ratio of 0.2:9.8. The structure of the compound was identified with the help of ¹³C-NMR, ¹H-NMR, heteronuclear multiple bond correlation (HMBC), heteronuclear multiple quantum coherence (HMQC), nuclear overhauser effect spectroscopy (NOESY) and correlation spectroscopy (COSY). The crude methanolic extract and various fractions of the aerial parts of the plant were also screened for anti-inflammatory activity. The EtOAc and *n*-hexane fractions of the plant showed moderate (44.8 and 40.9%, respectively) anti-inflammatory activity, while the rest of the fractions and crude extract showed low activity at concentration of 500 µg/ml against human neutrophils.

Key words: *Myrsine africana*, anti-inflammatory activity, indomethacin.

INTRODUCTION

Pakistan is endowed with the wealth of medicinal plants and has valuable heritage of herbal remedies. Like most developing countries, rural population of Pakistan still relies mainly on indigenous system of medicine for their health and health related matters (Khattak et al., 1985). Pakistan has a very rich flora due to its soil conditions and multiple ecological regions. Pakistan has about 6000 species of phanerogams. Estimates indicate that upto 700 plant species are used as medicinal and aromatic plants (Pei, 1992). According to a survey by Pakistan Forest Institute, 75 crude herbal drugs are extensively exported and more than 200 are locally traded in Pakistan. Eighty five percent of the crude herbs are

collected by the indigenous people from the forests (Rehman and Choudhary, 2003). Crude medicinal plant materials worth more than 2.3 million US \$ per year, are used in Pakistan. Large quantities of crude plants are exported to the international market (6 million US \$) although at low prices (Rehman and Choudhary, 2003).

In continuation of our previously reported work on medicinal plants (Bashir et al., 2010, 2011; Bashir, 2010), we select *Myrsine africana*, a herbaceous plant, belonging to the family *Myrsinaceae*, a large family of about 35 genera and nearly 1000 species, and widespread mainly in the tropical and subtropical regions (Nasir and Ali, 1979). Traditionally, the plant is used as fragrance in tea, spices, carminative, appetizer and flavoring agent. Its fruits are edible and locally used as an anthelmintic (Desta, 1995), for the treatment of diarrhea, rheumatism, toothache, pulmonary tuberculosis and relieving hemorrhage (Zhong, 1985). The alcoholic extract of twigs and leaves of the plant possesses significant inhibitory activities against walker intramuscular carcinosarcoma in rats (Kupchan, 1969). Preparations from the mixture of dried fruits and leaves of *M. africana* in water has shown 77% efficacy against *Haemonchus*, *Trichostrongylus* and *Oesophagostomum*

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Abbreviations: NMR, Nuclear magnetic resonance; TMS, tetramethylsilane; TLC, thin layer chromatography; CC, column chromatography; HMBC, heteronuclear multiple bond correlation; HMQC, heteronuclear multiple quantum coherence; HSQC, heteronuclear single quantum coherence spectroscopy; MHS, Modified Hank's solution;

spp. (Gathuma et al., 2004). The study previously done, revealed that plant contains flavonoids, benzoquinones, steroids and triterpenes (Manguro et al., 1996, 1997a; 1997b, 1997c, 2003; Li and McLaughlin, 1989). From the leaves methanolic extract, a new flavonol glycoside (quercetin 3-rhamnosyl galactoside) with other known flavonol glycosides have been reported (Manguro et al., 1997a). From the fruits of *M. africana* and *Maesa lanceolata*, two new natural benzoquinones: 2,5-dihydroxy-3-methyl-6-undecyl-1, 4-benzoquinone and 2-O-methyl-muketanin, respectively, were produced (Ogweno and Arot, 1996). Two novel flavonoids myrsinone A and B have been reported from the ethanolic extract of *M. africana*. Three new glycosides (myrsinoside A, B and (3 β , 16 α , 20 α)-3, 16,28-trihydroxyolean-12-en-29-oic-acid-3-{O- β -D-glucopyranosyl-(1-2)-O-[β -D-glucopyranosyl-(1-4)- α -D-arabinopyranoside]}) were produced from the ethanolic extract of the stem of the plant (Yan et al., 2008).

This manuscript deals with isolation and characterization of the olenane type triterpene with double bond at C-9/C-11. A compound similar to this but different in stereochemistry has been synthetically reported, but this compound has been isolated for the first time from natural source, and the final structure with complete nuclear magnetic resonance (NMR) data (Allan and Spring, 1955). The screening of the plant material for biological/pharmacological activity was also done in the present work.

MATERIALS AND METHODS

General experimental procedure

Jeol JMS 600 and HX 110 mass spectrometers with the data system DA 5000 was used to record HREI-MS. The ¹H-NMR spectrum was recorded in C₅D₅N on Bruker AMX-400 and AMX-500 NMR spectrometers with tetramethylsilane (TMS) as an internal standard using a UNIX operating system at 400 and 500 MHz, respectively. The ¹³C-NMR spectrum was recorded at 100 MHz on a Bruker AMX-500 NMR spectrometer in C₅D₅N. The [α]_D was recorded on Jasco P-2000 polarimeter. JASCO J-810 spectropolarimeter was used to record CD spectrum. Silica gel (230-270 mesh) was used for column chromatography (CC). Silica gel coated thin layer chromatography (TLC) card (GF-254, 20 × 20 cm, 0.25 mm thick, Merck) were used to check the purity of the compound and were observed under UV light (254 and 366 nm) and ceric sulphate was used as a spraying reagent.

Plant material

Aerial parts of *M. africana*, were collected from Hazara division, in December 2007 to January 2008. The plant was identified by Professor Dr. Habib Ahmad, Plant Taxonomist, Hazara University, Khyber Pukhtonkwha, Pakistan.

Extraction and fractionation

The plant material was chopped into small pieces and grinded to fine powder by using an electric grinder, after shade drying. These powdered materials (7.6 kg) were then soaked (twice) in

commercial grade methanol for 15 days at room temperature with occasional shaking. After 15 days, methanol soluble materials were filtered off. All the filtrates were concentrated under vacuum at 40 °C using a rotary evaporator till a blackish crude methanolic extract was obtained. After extraction, the crude methanolic extract of *M. africana* (Cr.MA) was suspended in distilled water (400 ml) and partitioned with *n*-hexane (3 × 400 ml), chloroform (CHCl₃) (3 × 400 ml), ethyl acetate (EtOAc) (3 × 400 ml) and butanol (BuOH) (3 × 400 ml) to yield *n*-hexane (50 g), CHCl₃ (45 g), EtOAc (255 g), BuOH (190 g) and aqueous (210 g) fractions, as per our reported procedure (Bashir et al., 2010). Fifty grams of Cr.MA was reserved for pharmacological/biological activities.

In vitro anti-inflammatory activity

Inflammation, a self-protective retort, induces physiological adaptations to remove pathogenic infections and reduce tissue damage (Roussin et al., 1997). Reactive oxygen species (ROS) are formed following the assemblage and activation of NADPH oxidase (phagocyte-specific enzyme). The process is initiated, during respiratory burst of non-mitochondrial oxygen (O₂) uptake by NADPH oxidase system, by production of superoxide anion (O⁻²) (Tan and Berridge, 2000). A water soluble tetrazolium salt (WST-1) is used in this study for the measurement of superoxide production by neutrophils which will be activated by opsonized zymosan, inducing phagocytic activation of neutrophils. This is a more sensitive and consistent technique among other existing techniques (Costantino et al., 1998).

Isolation of human neutrophils

Human neutrophils were isolated according to the procedure of Siddiqui et al. (1995). Fresh heparinized blood was collected from healthy volunteers. Equal volume of Modified Hank's solution (MHS, pH 7.4) was added to dilute the blood and left for 20 min at room temperature. The upper layer of leukocyte was collected and layered over Ficoll paque. The pellets obtained after centrifugation were resuspended with MHS. The cells were counted using an improved Neubaur chamber. The viability of the cells was 97% measured by Trypan Blue method.

Respiratory burst assay

The activity was performed according to our reported procedure (Bashir et al., 2010). The *in vitro* assay was based on the reduction of WST-1 in the presence of activated neutrophils. A total volume of 200 μ l MHS (pH 7.4), containing neutrophils (1.0 - 10⁴ / ml), WST-1 (250 μ M) and test samples was used to determine the anti-inflammatory activity. Neutrophils, buffer solution and WST-1 were added to the control. The samples were equilibrated at 37 °C by addition of opsonized zymosan A (15 mg/ml), prepared by mixing with human pooled serum, and the reaction was initiated. The absorbance of the reaction mixture was determined at 450 nm. For the positive control, the non-steroidal anti-inflammatory drug "indomethacin" was used which is widely used for the treatment of several inflammatory diseases. The percentage inhibitory activity showed by plant extracts was determined against blank and calculated as follows:

$$\text{Percentage inhibition} = 100 - [(\text{OD test compound}/\text{OD control}) \times 100]$$

RESULTS AND DISCUSSION

A new compound, named myrsinene, was isolated as

Table 1. ^1H and ^{13}C -NMR and chemical shifts of myrsinene (ppm, $\text{C}_5\text{D}_5\text{N}$, 500 and 100 MHz, respectively).

S/N	Multiplicity (DEPT)	^{13}C -NMR	^1H ($J = \text{Hz}$)
1	CH_2	35.3	1.12 m, 1.38 m
2	CH_2	28.0	1.21 m, 1.82 m
3	CH	78.1	3.43 (dd, $J = 10.5, 5.0$ Hz)
4	C	37.7	-
5	CH	55.9	0.81 m
6	CH_2	17.8	1.49 m, 1.61 m
7	CH_2	33.3	1.26 m, 1.37 m
8	C	39.4	-
9	C	158.4	-
10	C	38.2	-
11	CH	117	5.61 dd ($J = 8.0, 3.5$ Hz)
12	CH_2	33.9	1.51 m, 1.59 m
13	CH	49.1	1.15 m
14	C	39.2	-
15	CH_2	29.9	1.21 m, 1.83 m
16	CH_2	36.8	1.01 m, 1.34 m
17	C	36.0	-
18	CH	49.5	1.45 m
19	CH_2	41.6	1.36 m, 2.25 m
20	C	28.9	-
21	CH_2	37.9	1.69 m, 1.99 m
22	CH_2	38.2	1.53 m, 1.68 m
23	CH_3	30.0	0.89 s
24	CH_3	16.4	1.06 s
25	CH_3	21.5	0.98 s
26	CH_3	26.1	1.11 s
27	CH_3	15.6	0.94 s
28	CH_3	28.6	1.23 s
29	CH_3	33.3	0.97 s
30	CH_3	30.0	0.99 s

white powder from the EtOAc fraction of *M. africana*, eluting with EtOAc : *n*-hexane (0.2:9.8) as mobile phase on flash silica column. EI-MS of the compound showed M^+ at m/z 426. Its optical rotation, $\{[\alpha]_{\text{D}25} = +39.1$ (MeOH, $c = 0.4$) $\}$ indicated the presence of chiral centre in the compound. HR-EI-MS showed molecular ion peak at m/z 426.3862, corresponding to the formula $\text{C}_{30}\text{H}_{50}\text{O}$ (Cal. for $\text{C}_{30}\text{H}_{50}\text{O}$, 426.3861). The infrared (IR) spectrum showed absorptions at ν_{max} ; 3400 (OH), 2820 (CH).

^1H -NMR spectrum of the compound showed eight singlets due to methyl groups at δ 0.89, 0.94, 0.97, 0.98, 0.99, 1.06, 1.11 and 1.23, which were due to C-23, C-27, C-29, C-25, C-30, C-24, C-26 and C-28 methyl protons, respectively (Table 1). One proton signal at δ 3.43 (dd, $J_{3,2} = 10.5$ Hz, $J_{3,1} = 5.0$ Hz) was due to methine proton (H-3). Coupling constant values suggest the axial orientation of (H-3) proton. A downfield shift signal at δ -5.61 (dd, $J = 8.0, 3.5$ Hz) was assigned to the H-11. The ^1H -NMR data clearly indicated that the compound is

triterpene (Chatterjee and Chakroborti, 1980).

^{13}C -NMR spectrum (BB, DEPT) displayed 30 carbon resonance including eight methyl, ten methylene, five methine and seven quaternary carbons (Table 1). The structure of compound (Figure 1) was further confirmed from 2D-NMR spectroscopy (HSQC, HMBC, COSY and NOESY) data. Position of the double bond was assigned with the help of ^{13}C -NMR values and HMBC interactions (Figure 2). Methyl protons of C-25 and C-26 displayed HMBC correlations with C-9 (158.4), which confirmed the position of double bond at C-9 and C-11. Circular dichroism (CD) spectrum of the compound indicate an intense positive cotton effect at 280 nm. ^1H - ^{13}C connectivities were determined by using HSQC interactions. Stereochemistry of the compound was deduced by the help of biosynthetic pathway (Shiojima et al., 1995), ^1H - ^1H coupling constant values and nuclear overhauser effect spectroscopy (NOESY) correlations (Figure 3).

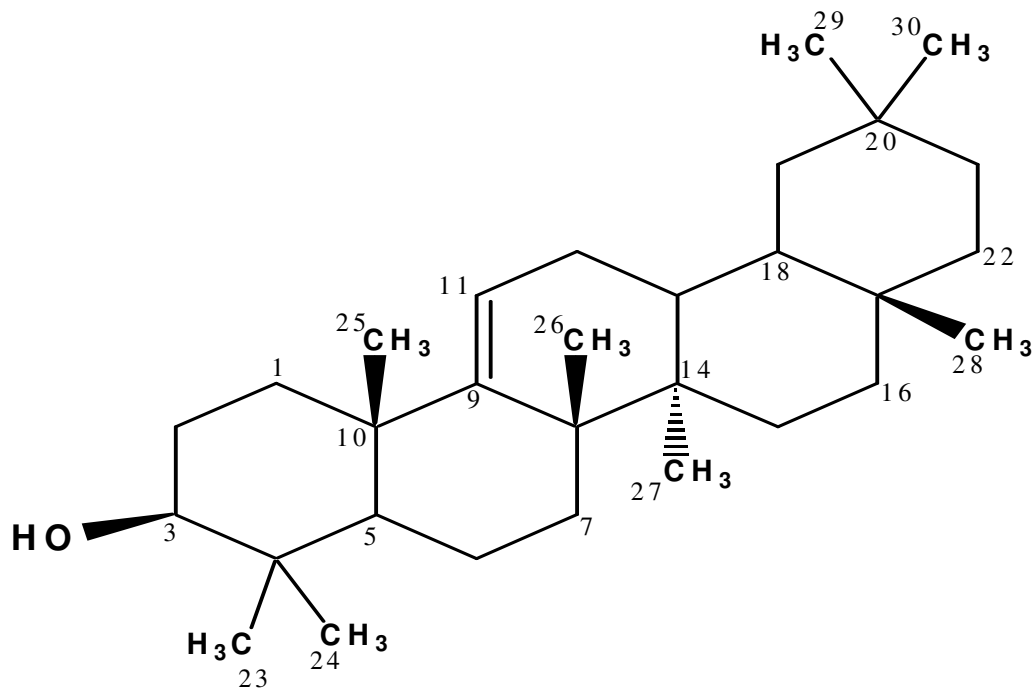


Figure 1. Structure of myrsinene.

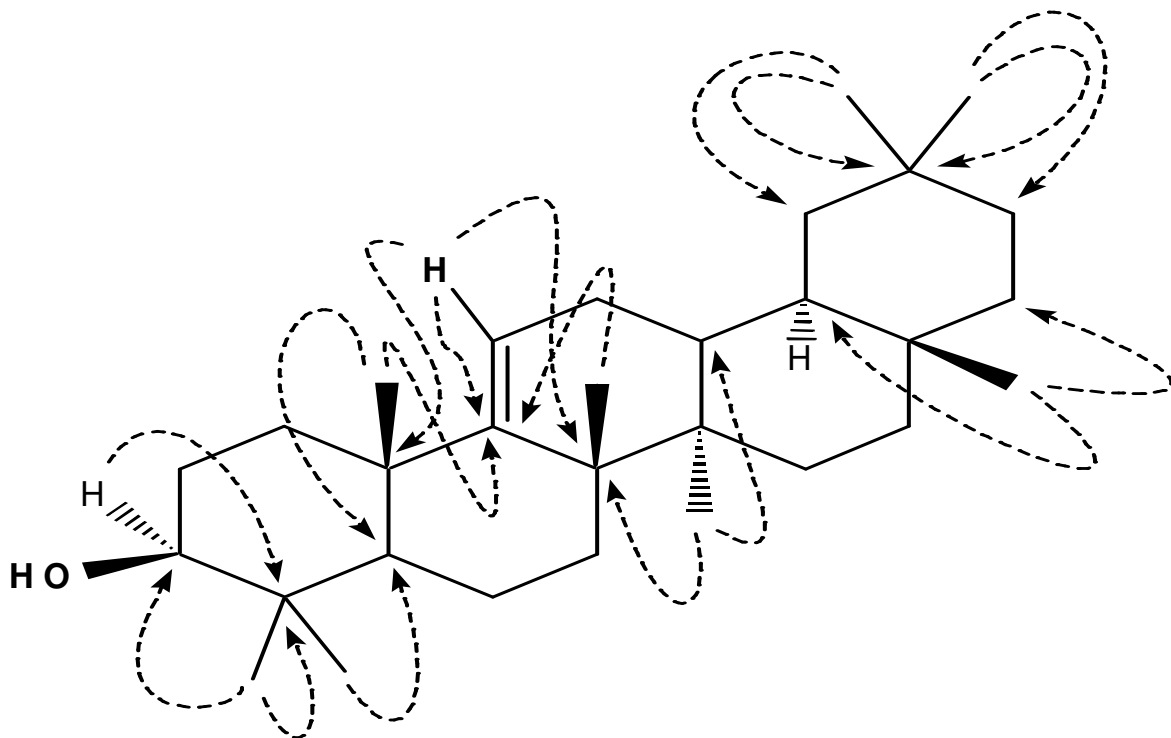


Figure 2. Key HMBC correlations in myrsinene.

Anti-inflammatory activity

The main causes of the inflammation are the immune

system reaction to infection, malignancy, cellular changes, environmental agents and injury. Inflammation is a key mark in autoimmune disease. In conditions like

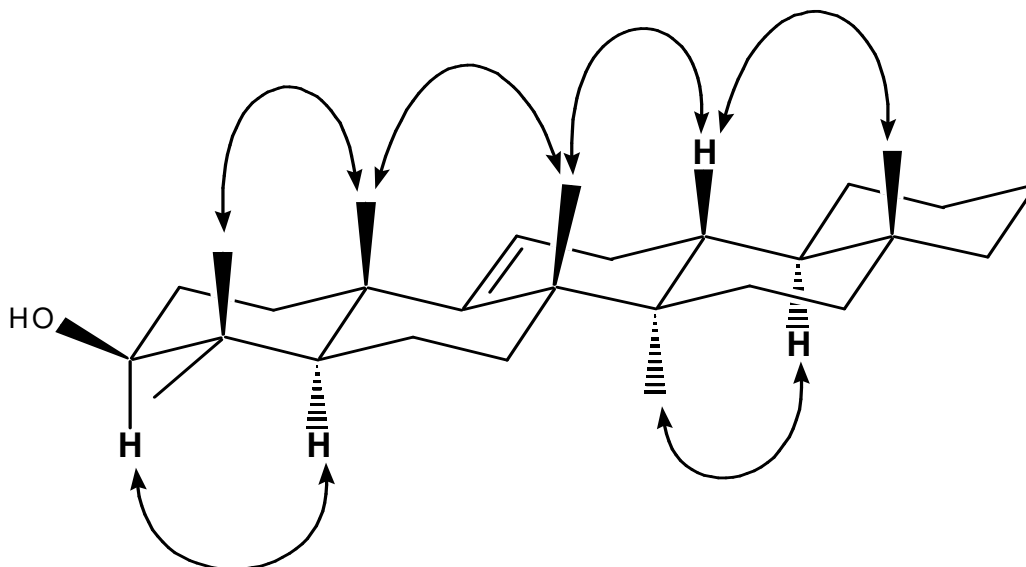


Figure 3. Key NOESY correlations in myrsinene.

Table 2. Anti-inflammatory activity of the Cr.MA and various fractions of *M. africana*.

Test sample	Percentage inhibition at 500 µg/ml
Cr.MA	38.5
<i>n</i> -hexane	40.9
CHCl ₃	37.6
EtOAc	44.8
BuOH	33.1
Aqueous	29.7
* Indomethacin	98.0

*Standard drug; EtOAc, ethyl acetate; Cr.MA, crude methanolic extract of *M. Africana*; BuOH, butanol.

Hashimoto's thyroiditis, the disease process is due to inflammation, while in some other conditions like Crohn's disease, the cause of inflammation is the diseased condition (Elaine, 2006). As the herbs possess anti-inflammatory characteristics, we screen crude methanolic extract and various fractions of the plant for possible anti-inflammatory activity. The results obtained are shown in Table 2, which reveals that *n*-hexane and EtOAc fractions of the plant showed moderate (40.9 and 44.8%, respectively) anti-inflammatory activity at concentration of 500 µg/ml. The rest of the fractions and crude methanolic extract showed low anti-inflammatory activity at the same concentration.

Conclusion

The present study reveals that the EtOAc and *n*-hexane fractions of the plant (*M. africana*) showed moderate

(44.8 and 40.9%) anti-inflammatory activity against human neutrophils and needs isolation of the pure constituent which possesses anti-inflammatory actions.

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