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Full Length Research Paper

Insecticidal, brine shrimp cytotoxicity, antifungal and nitric oxide free radical scavenging activities of the aerial parts of *Myrsine africana* L.

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The crude methanolic extract and various fractions derived from the aerial parts of *Myrsine africana* were screened *in vitro* for possible insecticidal, antifungal, brine shrimp lethality and nitric oxide free radical scavenging activities. Low insecticidal activity (20 %) was shown by chloroform ($CHCl_3$) and aqueous fractions against *Tribolium castaneum* and *Rhizopertha dominica*, respectively. Good cytotoxic activity (66.66 %) was shown by the *n*-hexane fraction of the plant at 1000 µg/ml. The rest of the fractions showed low lethality at higher doses. No antifungal activity was observed for the crude extract and fractions screened against various fungal strains. The plant crude extract and fractions showed a concentration dependent nitric oxide free radical scavenging activity.

Key words: *Myrsine africana*, insecticidal, brine shrimp lethality, antifungal and nitric oxide free radical scavenging assay.

INTRODUCTION

The ability of higher plants to produce valuable natural products with interesting bioactivities has been reported in the past decades (Liu et al., 2007; Morita et al., 2006; Pepeljnjak et al., 2005; Tiew et al., 2003). *Myrsine africana*, locally called Babrang belongs to the family Myrsinaceae. This is a large family of about 35 genera and nearly 1000 species, which is widely distributed mainly in the tropical and subtropical regions (Nasir and Ali, 1988). *M. africana* is traditionally used as fragrance in tea, spices, appetizer, carminative and as a flavoring agent. The fruits of the plant are locally used as an anthelmintic and are edible (Zabta et al., 2003; Kokwaro 1993; Beentje 1994; Desta 1995). The fruits can also be used for the treatment of diarrhea, rheumatism, tooth-ache, pulmonary tuberculosis and relieving hemorrhage (Zhong, 1985). A significant inhibitory activity against walker intramuscular carcinosarcoma in rats was reported for the alcoholic extract of twigs and leaves of the plant (Kupchan et al., 1969). Preparations from the mixture of dried fruits and leaves of *M. africana* in water showed 77% efficacy against *Haemonchus*, *Trichostrogylus* and *Oesophagostomum* spps. (Gathuma et al., 2004). Leaves and fruits extracts of *M. africana* showed no effects against *Haemonchus contortus* in sheep (Githiori et al., 2002). Lethal effect was shown by the ethanolic extract of the fruits against tape worms, while a marked purgative activity in albino rats was observed for the ethereal, alcoholic and aqueous extracts of the plant (Kakrani and Kalyani 1983). A non specific agglutination reaction of the seeds extract of *Rapanes guinensis* Aubl with human bloods have also been reported (Schertz et al., 1960). The anti-fertility activity of *Embelia ribes* was also reported (Arora et al., 1971). From the ethanolic extract of the roots of *M. Africana*, two compounds emodin and 2-hydroxychrysophanol have been reported as cytotoxic components. Nepodin and 5-methoxy-7-

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hydroxyphthalide were also isolated from the plant but not as effective cytotoxic agents as the earlier ones (Xiao-hua and McLaughlin, 1989). Embelin isolated from *Rapanea melanophloeos* showed larvicidal effect against *Aedes egyptii* and as an anti-feedant to *Schistocerca gregaria* (Midiwo et al., 1995). Saponins isolated from the leaves of *Maesa lanceolata* exhibited viricidal, haemolytic and anthelmintic activities (Apers et al., 2001; Bagalwa and Chifundera 2007). The major compounds reported from *M. lanceolata, R. melanophloeos, M. africana* and *Embelia schimperi* are 2,5-dihydroxy alkyl and 2,5-dihydroxyalkylbenzoquinone derivatives, maesaqui-none, acetylmessaquinone, maesanin, embelin and repanone. The first three compounds are found only in *M. lanceolata* and the latter are found in the other species beside minor and medium level compounds found in these four species (Midiwo et al., 1988). Based on the reported literature, *M. Africana* was screened for possible pharmacological/ biological activities and then the ethyl acetate (EtOAc) fraction was subjected to column chromatography for isolation of compounds.

MATERIALS AND METHODS

Plant material

Aerial parts of *M. africana* (*Myrsinaceae*), were collected from the mountainous area of Hazara division, during winter 2007. The plant material was identified with the help of available literature.

Extraction

The shade dried plant materials were chopped into small pieces and grinded to fine powder by using electric grinder. The powdered plant material (7.6 kg) was soaked in commercial grade methanol for 15 days at room temperature with occasional shaking. After 15 days, methanol soluble materials were filtered off. All filtrates were combined and concentrated, under vacuum at 40°C using a rotary evaporator till a blackish crude methanolic extract of about 800 g was obtained.

Fractionation

The crude methanolic extract (750 g) was suspended in distilled water (400 ml) and partitioned with *n*-hexane (3 x 400 ml), $CHCl_3$ (3 x 400 ml), EtOAc (3 x 400 ml) and buthanol (BuOH) (3 x 400 ml) to yield *n*-hexane (50 g), $CHCl_3$ (45 g), EtOAc (255 g), BuOH (190 g) and aqueous (210 g) fractions. About 50 g of crude methanolic extract was reserved for other pharmacological/biological screenings.

Insecticidal activity

The insecticidal activity of the crude methanolic extract and various fractions were determined according to the reported procedure of Ahmad et al. (2009). Stock solution was prepared by dissolving 200 mg of each test sample in 3 ml of volatile organic solvent (methanol). The test solution was then applied to filter papers (90 mm diameter). After drying, each filter paper was placed in the separate Petri dish along with 10 adults of each *Tribolium castaneum, Rhyzopertha dominica* and *Callosbruchus analis.* Permethrin (235.71 µg/cm^2) and methanol were used as positive and negative controls, respectively. All these were kept without food for 24 h after which mortality count was done.

Brine shrimp cytotoxicity

The reagents and material used in the experiment were; test samples (crude extract and fractions), *Artemia salina*, sea salt (38 g/L of distilled water with pH 7.4), tray for hatching of eggs, lamp for attraction of brine shrimp larvae, micro pipette (of different ranges of 10 to 1000 µl), vials, methanol and distilled water. The cytotoxicity of the test samples was performed as per the reported procedure of (Ahmad et al. 2009).

Hatching technique

Hatching tray of 22 x 32 cm was half filled with filtered brine solution and 50 mg of shrimp eggs were sprinkled into it. The tray was incubated for 24 h at 37°C. After incubation, the eggs were hatched and the test samples were applied to find its cytotoxicity.

Sample preparation

Each test sample (10 mg) was dissolved in 1 ml of methanol to prepare a stock solution. Different concentrations of extract (1000, 100 and 10 µg/ml) from the stock solutions of the test samples were loaded to separate vials. The vials were placed in hood for an hour to evaporate the organic solvent. After two days of hatching and maturation, 10 larvae were transferred, with the help of pastuer pipette, to separate vials containing test samples. The volume of each vial was increased to 5 ml with seawater. Etoposide (LD_{50} = 7.465 µg/ml) and methanol were used as positive and negative controls, respectively. All the vials were incubated at 26 ± 1°C for 24 h. The data were analyzed with a Finney computer program to determine LD_{50} values with 95% confidence interval.

Antifungal activity

The crude extract along with fractions was screened against various fungal strains: *Aspergillus niger, Aspergillus flavus, Penicillium notatum, Fusarium oxysporum, Triticum harzianum* and *Rhizopus stolonifer.* The activity was performed by the reported procedure of Ahmad et al., 2009. A stock solution of each test sample at 24 mg/ml was prepared in sterile (autoclaved) dimethyl sulfoxide (DMSO, Merck). Sabouraud dextrose agar (SDA, Sigma-Aldrich, Germany) was prepared by mixing 32.5 g SDA in 500 ml distilled water and was mixed thoroughly with magnetic stirrer. Then, 4 ml amount was dispensed into screw cap tubes, which were autoclaved at 120°C for 15 min and then cooled to 50°C. The non-solidified SDA media was mixed with stock solution (66.6 µl) giving the final concentration of 400 µg of the extract per ml of SDA. Tubes were then allowed to solidify in the slanted position at room temperature. Each tube was inoculated with a piece (4 mm diameter) of inoculums removed from a 5 to 7 day old culture of fungi for non-mycelial growth; an agar surface streak was employ-yed. Other media supplemented with DMSO and reference anti-fungal drugs served as negative and positive control, respectively. On the 7th day, the visible non mycelial linear growths (mm) of the microorganisms were measured. The percent growth inhibition was calculated with reference to the standard drug.

Antioxidant activity

Sodium nitroprusside in aqueous solution at physiological pH,

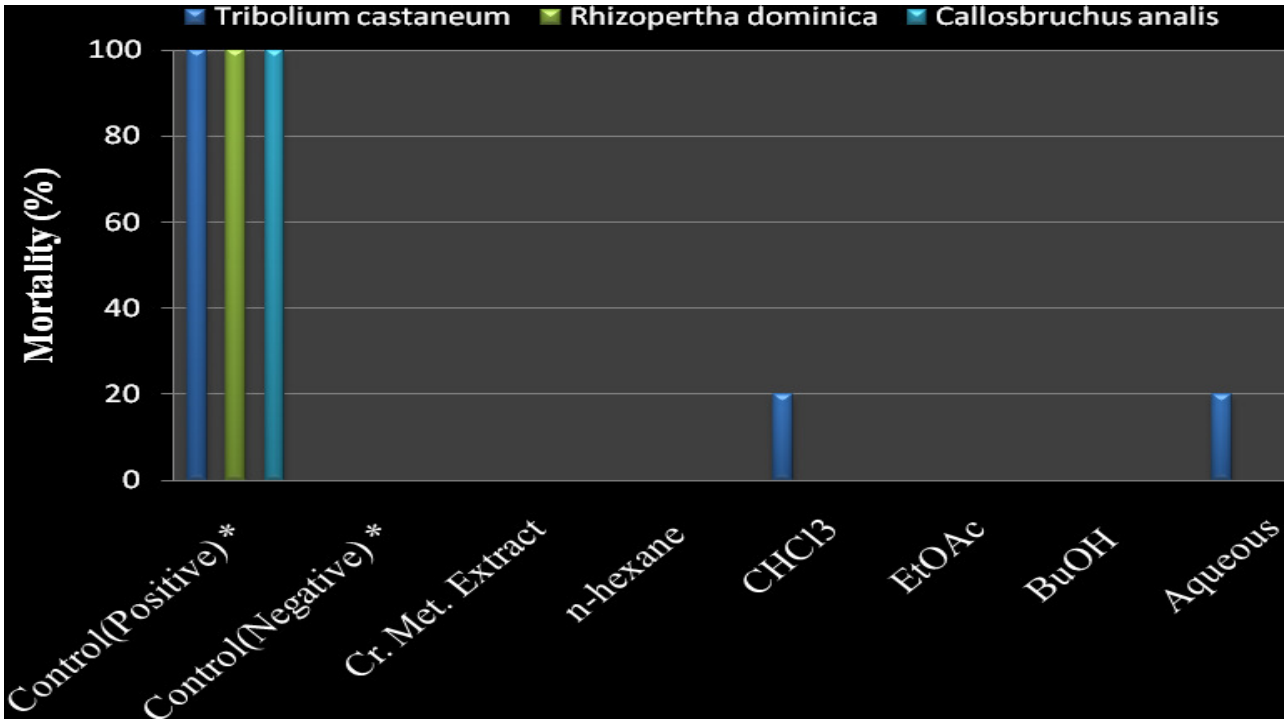

Figure 1. Insecticidal activity of crude extract and various fractions of *M. africana* against the selected insects. * Permethrin at 235.9 µg/cm^2 was used as standard drug in the positive control.

spontaneously generate nitric oxide to produce nitrite ion. These nitrite ions on reaction with sulphanilic acid produce a p-diazonium salt. Napthylethylenediaminedihydrochloride (0.1% w/v) on reaction with p- diazonium salt, formed a pink complex of Azo dye and the absorbance of the pink chromophore was measured at 570 nm.

Requirements

The following were used: 96-well microtiter plate reader (UV region), 96-well micro plate, multi-chamber micropipettes, test samples (crude extracts and its various fractions), sodium nitroprusside (10 mM) (Sigma Co), sulphanilic acid (0.33 % in 20 % acetic acid) (Sigma Co), [N-(1-napthyl) ethylenediaminedihydrochloride] (0.1 % in H2O)(Sigma Co)), phosphate buffer (10 mM) (pH = 7.4), vitamin C as positive control and DMSO as negative control.

Procedure

The antioxidant activity was performed by following the procedure: stock solution (3 mg/ml) of the test samples was prepared in DMSO. From this stock solution, by dilution, different concentrations of the test samples (0.3, 0.6, 0.9, 1.2 and 1.5 mg/ml) were prepared. 10 µl from each concentration of the test sample were taken in microtiter plate and 20 µl of phosphate buffer was added to it. Then 70 µl of sodium nitroprusside was added. The microtiter plate was then incubated at 20 to 25°C for one and half hours. The plate was well shaken after incubation and 50 µl of sulphanilic acid was added. The absorbance (pre-absorbance) at 570 nm was taken. After pre-reading, 50 µl of [N-(1-napthyl) ethylenediaminedihydrochloride], was added, shaken well and the final reading was take. Similarly, vitamin C and DMSO were run as positive control and blank, respectively.

RESULTS AND DISCUSSION

Insecticidal activity

The crude extract and fractions of *M. africana* were screened for insecticidal activity, using impregnated filter paper method (Tabassum et al., 1997), against three different insect species; *T. castaneum, R. dominica* and *C. analis.* The results presented in Figure 1, revealed that crude extract along with other fractions of the plant, showed no activity against the tested insects except $CHCl_3$ and aqueous fraction which showed low insecticidal activity (20%) each against *T. castaneum* and *R. dominica*, respectively. Permithrine (copex) was used as positive control, while the organic solvent (methanol) was treated as negative control. The positive control showed 100% mortality, while the mortality rate was 0% in the case of the negative control. From the results, it can be concluded that *M. africana* has no insecticidal property.

Brine shrimp lethality

The brine shrimp lethality test was performed to assess the toxicity of *M. africana*. The test samples (crude and fractions) were used in concentrations of 10, 100 and 1000 µg/ml. Results for the lethality were noted in term of deaths of larvae, compared with etoposide (standard drug). The calculated LD_{50} value for the standard drug

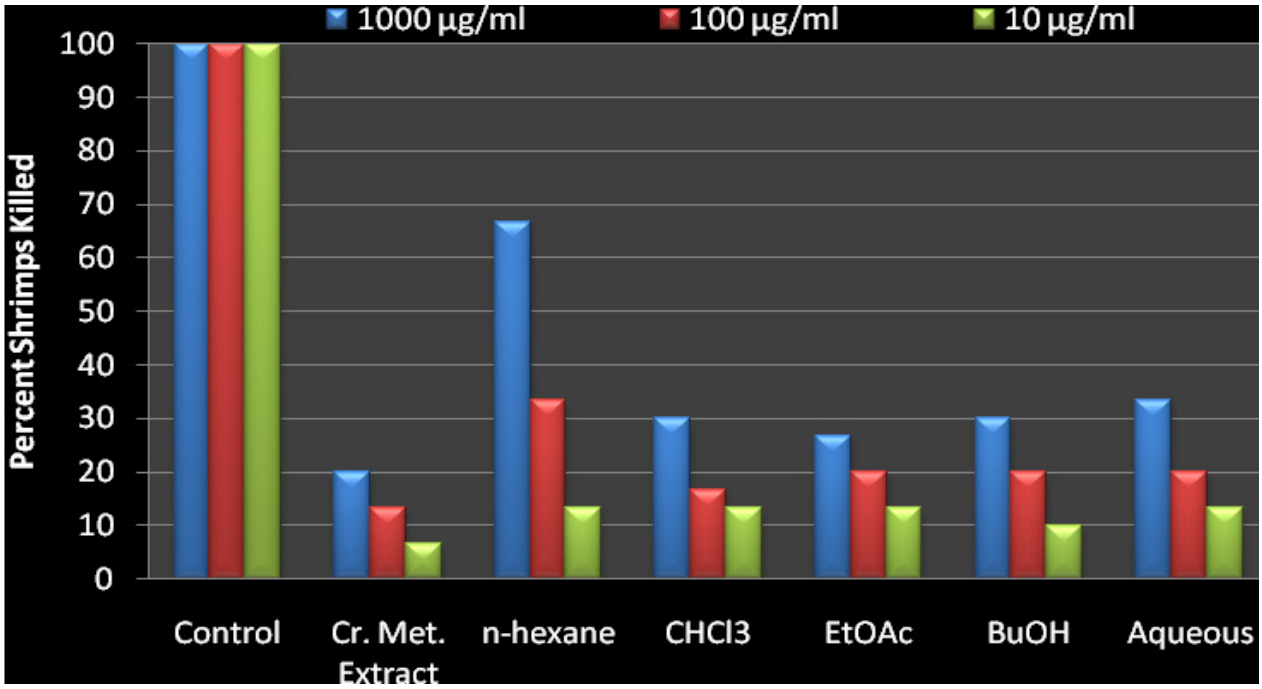

Figure 2. Brine shrimp lethality of crude extract and various fractions of *M. africana.* *Etoposide at 7.4625 µg/ml was used as the standard drug.

was 7.4625 µg/ml. The crude extract of the plant showed low cytotoxic activity (20.00, 13.33 and 6.66%) against the experimental shrimps, at 10, 100 and 1000 µg/ml, respectively. Different fractions exhibited varied cytotoxic activities. The *n*-hexane fraction showed good cytotoxicity (66.66%) at 1000 µg/ml, while low activity (33.33 and 13.33%) was observed at 100 and 10 µg/ml, respectively. The LD_{50} value calculated was 30.344 µg/ml for *n*-hexane fraction. The $CHCl_3$ showed low activity at all concentrations (30.0, 16.66 and 13.33% at 1000, 100 and 10 µg/ml, respectively). The EtOAc fraction showed low brine shrimp lethality at respective test concentrations that was: 26.66, 20.00 and 13.33% at 1000, 100 and 10 µg/ml, respectively. Similarly, the BuOH fraction of the plant showed low cytotoxic activity (30.0, 20 and 10.0%) at respective test concentrations. The aqueous fraction screened at the end of the experiment did not show any significant cytotoxic activity against the tested shrimps. The maximum cytotoxicity was 66.66% as shown by the *n*-hexane fraction of the plant at higher concentration. The cytotoxic results of the crude extract and all fractions are presented in Figure 2. From the results, it can be concluded that the *n*-hexane fraction of the plant contains cytotoxic constituents.

Antifungal activity

Persistent opportunistic fungal infections have become an important factor for morbidity and mortality in immunocompromised patients (Bodey et al., 1992). The majority of the fungal infections are mainly caused by the *Aspergillus* and *Candida* species. Recent trends in epidemiology have pointed out a shift towards infection caused by *Aspergillus* and non-albicans *Candida* (Groll et al., 1996). The test samples were screened for antifungal activity against *A. niger, A. flavus, P. notatum, F. oxysporum, T. harzianum* and *R. stolonifer* using agar tube dilution method. Amphotericin B and miconazole were used as standard drugs. The crude methanolic extract and all fractions of plant failed to show antifungal effect against *A. niger, P. notatum* and *R. stolonifer* as shown in Table 1. Low antifungal activity was observed for the crude extract (10%), *n*-hexane (10%), $CHCl_3$ (15%) and BuOH (5%) against *A. flavus*. In addition, the $CHCl_3$ and aqueous fractions showed low inhibiting effect of 15% and 24% respectively, against *F. oxysporum*; while, crude extract and other fractions were inactive against the tested fungal specie. Similarly the *n*-hexane, $CHCl_3$ and BuOH fractions of the plant showed low antifungal activity against *T. harzianum*. The rest of the fractions and crude extract were unable to inhibit the growth of the tested fungal species. The results showed that *M. africana* had no significant antifungal activity against the tested fungal species.

Nitric oxide (NO) free radical scavenging activity

Free radicals are constantly produced in the living system,

Table 1. Antifungal activity of the crude extract and various fractions of *M. africana*.

Fungi	Percent inhibition (%)							
	Negative control	Positive control	Crude extract	*n*-hexane	$CHCl_3$	EtOAc	BuOH	Aqueous
A. niger	0	100	0	0	0	0	0	0
A. flavus	0	100	10	10	15	0	5	0
P. notatum	0	100	0	0	0	0	0	0
F. oxysporum	0	100	0	0	15	0	0	24
T. harzianum	0	100	0	8	12	0	16	0
R. stolonifer	0	100	0	0	0	0	0	0

Table 2. Nitric oxide free radical scavenging activity of the crude extract and fractions of *M. africana*.

Concentration of sample (mg/ml)	Percent nitric oxide free radical scavenging activity of samples (%)					
	Crude extract	*n*-hexane	$CHCl_3$	EtOAc	BuOH	Aqueous
0.3	32.27	23.49	28.13	22.03	26.74	35.36
0.6	38.04	27.47	34.47	34.3	30.24	45.28
0.9	45.69	33.73	36.82	39.52	33.98	49.91
1.2	49.17	36.17	39.26	43.69	38.94	52.68
1.5	53.05	41.94	44.65	49.59	43.65	55.36

Vitamin C was used as standard at 47.78 µg/ml.

which can cause an extensive damage to bio-molecules and tissues thereby causing various diseases like extensive lysis and degenerative diseases (Ames et al., 1993). For reduction of these oxidative damages, many synthetic drugs are used but their side effects cannot be ignored. The consumption of the natural food products and traditional medicines containing antioxidant constituents are alternative ways to avoid the side effects. Recently, from different natural sources, various natural antioxidants have been reported (Peng et al., 2003; Bakar et al., 2009; Lin et al., 2009).

Due to the importance of the natural antioxidants, the crude methanolic extract and various fractions of *M. africana* at different concentrations (0.3, 0.6, 0.9, 1.2 and 1.5 mg) were screened for possible nitric oxide free radical scavenging assay. The results obtained are shown in Table 2 and it indicates that the crude methanolic extract (32.27%) and aqueous fraction (35.36%) of the plant possessed moderate NO free radical scavenging activity, while low activity was observed for other fractions including *n*-hexane (23.49%), $CHCl_3$ (28.13%), EtOAc (22.03%) and BuOH (26.74%) at 0.3 mg/ml. The NO free radical scavenging activity was increased by increasing the concentration of the test samples and at 0.6 mg/ml, the crude extract along with four other fractions showed moderate activity, that is, 38.04% for crude extract, 34.47% for *n*-hexane, 34.30% for EtOAc, 30.24% for BuOH and the aqueous fraction of the plant showed 45.28% activity. At 0.9 mg/ml, all the test samples showed moderate NO free radical scavenging activity in the order of: aqueous (49.91%) > crude extract (45.69%) > EtOAc (39.52%) > $CHCl_3$ (36.82%) > BuOH (33.98%) > *n*-hexane (33.73%). Similarly, the activity was performed at 1.2 mg/ml, indicating that the crude extract along with other fractions showed moderate NO free radical scavenging activity in the order of: aqueous (52.68%) > crude extract (49.17%) > EtOAc (43.69%) > $CHCl_3$ (39.26%) > BuOH (38.94%) > *n*-hexane (36.17%). The activity was also performed at 1.5 mg/ml. The aqueous fraction and crude extract showed the highest activity (55.36 and 53.05%), respectively, and the rest fractions: EtOAc (49.59%), $CHCl_3$ (44.65%), BuOH (43.65%) and the *n*-hexane showed 41.94% NO free radical scavenging activity. From the results, it can be concluded that the plant crude extract and fractions possess concentration dependent nitric oxide free radical scavenging activity that necessitates further work to isolate the active molecules from *M. africana*.

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