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Phytochemical and antimicrobial activity of *Monechma ciliatum* (black mahlab) seed extracts

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Abbreviations:
Sa: *Staphylococcus aureus*;
Pa: *Pseudomonas aeruginosa*;
Kp: *Klebsiella pneumoniae*;
Ca: *Candida albicans*;
An: *Aspergillus niger*;
Af: *Aspergillus flavus*.

ABSTRACT

Background & Aim: The purpose of present study was to examine and validate the folk's claim of inhalation of *Monechma ciliatum* seeds powder by traditional African natives for cold and allergic conditions, so as to formulate a useful therapeutic agent to respiratory tract infections.
Experimental: Different extracts were bioassayed *in- vitro* for their bioactivity to inhibit the growth of pathogenic bacteria and fungi. Bacteria and fungi selected in the study were common pathogens for respiratory tract infections.
Results & Discussion: *Klebsiella pneumoniae* was highly sensitive to the hydroalcoholic extract (maceration by 70% ethanol 30% water) and moderately sensitive to the extract (maceration by water). *Staphylococcus aureus* was found to be moderately sensitive to the hydroalcoholic extract (maceration by 70% ethanol 30% water). *Pseudomonas aeruginosa* was resistance to all extract used. In addition, all fungi used were found to be resistance to all extracts. Phytochemical screening test results of seeds of *Monechma ciliatum* to detect the major constituents showed the presence of flavonoids, tannins, triterpens, and anthraquinones. The findings of study support the traditional uses of the plant in the therapy of respiratory tract infections.
Recommended applications/industries: The extracts of black mahlab could be used in future direction as alternative therapeutic agents for the treatment of human's respiratory tract infectious diseases caused by *Klebsiella pneumoniae* and *Staphylococcus aureus.*

1. Introduction

According to World Health Organization (WHO), interest in traditional systems of medicine and, in particular, herbal medicines, has increased substantially in both developed and developing countries over the past two decades (WHO, 2003). A WHO survey indicates that about 70 - 80% of the world population, particularly in the developing countries; rely on non-conventional medicines mainly of herbal sources in their primary healthcare. WHO has described traditional medicine as one of the surest means to achieve total healthcare coverage of the world's population. In pursuance of its goal of providing accessible and culturally acceptable health care for the global population, WHO has encouraged the rational use of traditional plant based medicines by member

states and has developed technical guidelines for the assessment of herbal medicine (Okunlola *et al*., 2003).

Infection remains the main cause of morbidity and mortality in man, particularly in under-developed areas where it is associated with poverty and overcrowding. In the developed world increasing prosperity, universal immunization, and antibiotics have reduced the prevalence of infectious disease. However, antibiotic-resistant strains of bacteria as well as viruses and new diseases including Human Immunodeficiency Virus (HIV) infection and variant Creutzfeldt- Jackob Disease variant (vCJD) have emerged. The common respiratory tract infections are influenza (caused by *influenzae* virus, its main complications the secondary bacterial infection by *Staphylococcus aureus* having a mortality of up to 20%), Pneumonia (usually caused by bacteria *Staphylococcus aureus, Legionella pneumoniae, Coxiella burnentii, Pseudomonas aeruginosa, Pneumocystis carinii, Klebsiella pneumoniae*, and anaerobic organism) and Sinusitis infections are usually caused by *Streptococcus pneumoniae* and *Haemophilus influenzae* (Kumar & Clark, 2003). Deep fungus infections have increased rapidly in recent years. Most fungus infections are caused by candida germ, mainly *Candida albicans*. The death rate from these infections can reach to 30-60%. Unfortunately, few anti-fungus medicines are available for treating fungus infections, not to mention that most of them have serious side effects (Sheng *et al*., 2012).

Monechma ciliatum is a plant distributed throughout tropical Africa, as wild and also cultivated in savanna (Fig.1). In Sudan, it is locally known as 'El-Mahlab El-Aswad', or 'El-Mehbab'. It grows in western and southwestern parts of Sudan, especially in the Nuba Mountains and Gabel Mara area. In Nigeria it is known as 'Bakin mata' in Hausa language. In Sudan the dried seeds (Fig. 2) are used as a scent preparation, also reported that in Sudan the entire dried plant is used for diarrhea and vomiting (Uguru & Evan, 2002). It is used as antispasmodic, and the aqueous extract used as laxative. The seeds are used as an effective laxative; it is further used in traditional Sudanese fragrances, lotions, and other cosmetics used for wedding preparation and childbirth rituals such as 'Dilka', 'Khumrra' (Mariod *et al*., 2009).

Monechma ciliatum is a plant used in folk medicine by traditional midwives to induce labor and menses in the northern parts of Nigeria. It is reported that the dried leaves and seeds are powdered and burnt as an inhalation for treatment of cold. In Edo and Delta states of Nigeria, the plant is used as anthelmintic and anti-dysenteric (Uguru & Evan, 2002).

The seeds of plant contain hydrocarbons and fatty acids (Ayoub, 1981). Recently, the seeds were screened by Mariod et al. (2009); they found fatty acids (palmitic, stearic, linoleic, and others); tocopherols; protein; and elements (K, Ca, Mg, Al, Pb, Ni, Mn, Cu, Cr, Co, and Fe). The paucity of antimicrobial and chemical data of this herbal medicine prompted an investigation into its antimicrobial activity. Therefore, the present study was planned to find out the antimicrobial activity and their efficacy against different bacterial and fungal infections.

Fig. 1. *Monechma ciliatum* (A wild Sudanese medicinal plant)

Fig. 2. Seeds of *Monechma ciliatum*

2. Materials and Methods

2.1. Plant materials and chemicals

The study was done at the Medicinal and Aromatic Plant Research Institute (MAPRI) of Khartoum, Sudan and Omdurman Islamic University, Omdurman, Sudan. Black mahlab seeds samples used in this study were collected from Nubia Mountains (South Kordofan state, Sudan). The seeds were identified in Department of pharmacognosy Alnaileen University-Sudan, and authenticated at the Medicinal and Aromatic Plant Research Institute (MAPRI) of Khartoum-Sudan. The seeds were collected between September and December (autumn), separated, and dried under shade. The dried seeds were stored in a covered bottle at room temperature until required for use.

Agar: HiMedia Laboratories Pvt. Ltd, India. Ampicillin: Bristol – Mayers Squibb, USA. Clotrimazole: General Medicine Co., Sudan. Ethanol: Scharlau Chemie S.A, Spain. Gentamicin: Roussel, UK. Methanol: BDH laboratory, UK. Nystatin: Sigma Medical Company, USA. Sabouraut agar: HiMedia Laboratories Pvt. Ltd, India. Standard bacteria and fungi: Three types of standard bacteria namely *Staphylococcus aureus* (ATCC 25923), *Klebsiella pneumoniae* (ATCC 53657) and *Pseudomonas aeruginosa* (ATCC 27853) were obtained from MAPRI. Three types of standard fungi namely *Aspergillus fumigates* (ATCC13073), *Aspergillus niger* (ATCC 9763) and *Candida albicans* (ATCC 7596) were obtained from MAPRI.

2.2. Preparation of the extracts

2.2.1. Maceration method by using water, methanol or ethanol as a solvent system. The dried seeds for *Monechma ciliatum* were reduced to a coarse powder by using mesh No. 35; 20 g of the coarse powder weighed, and transferred to flasks containing 400 mL of distilled water, methanol [30, 50, 70% (v/v)] or ethanol [30, 50, 70% (v/v)] separately. The flasks were allowed for 24h at room temperature, with occasional shaking. Then, the extract of each flask was passed through a cotton wool and the solid residue (marc) pressed (screw press). The strained and expressed liquid obtained was mixed, put for 12 h for clarification and filtrated. Finally, the filtrates were evaporated to reduce the solvent volume using rotary evaporator under reduced pressure at 40 ºC and placed in a Petri dish, and left to dry to constant weight.

2.2.2. Infusion method. The dried seeds for *M. ciliatum* were reduced to a coarse powder by using mesh No. 35; 20 g of the coarse powder weighed, and transferred to flasks. 400 mL of cold and hot boiled water added to two flasks separately. The flasks were allowed for 24 h at room temperature, with occasional shaking. Then, the extract of each flask was passed through a cotton wool and the solid residue (marc) pressed (screw press). The strained and expressed liquid obtained was mixed, put for 12 h for clarification and filtrated. Finally, the filtrates were evaporated to reduce the solvent volume using rotary evaporator under reduced pressure at 40 ºC and placed in a Petri dish, and left to dry to constant weight

2.2.3. Decoction method. The dried seeds for *M. ciliatum* were reduced to a coarse powder by using mesh No. 35; 20 g of the coarse powder weighed, and transferred to 4 flasks containing 200 mL of boiled water and allowed to stand for 5, 10, 15, and 20 min respectively. The flasks were allowed for 24 h at room temperature, with occasional shaking. Then, the extract of each flask was passed through a cotton wool and the solid residue (marc) pressed (screw press). The strained and expressed liquid obtained was mixed, put for 12 h for clarification and filtrated. Finally, the filtrates were evaporated to reduce the solvent volume using rotary evaporator under reduced pressure at 40 ºC and placed in a Petri dish, and left to dry to constant weight.

2.2.4. Soxhlet extraction. The dried seeds for *M. ciliatum* were reduced to a coarse powder by using mesh No. 35; 20 g of the coarse powder weighed, and transferred to three Soxhlet containing 200 mL of methanol 70% (v/v), ethanol 70% (v/v) and chloroform separately, and allowed for 10 h. The flasks were allowed for 24 h at room temperature, with occasional shaking. Then, the extract of each flask was passed through a cotton wool and the solid residue (marc) pressed (screw press). The strained and expressed liquid obtained was mixed, put for 12 h for clarification and filtrated. Finally, the filtrates were evaporated to reduce the solvent volume using rotary evaporator under reduced pressure at 40 ºC and placed in a Petri dish, and left to dry to constant weight.

2.5. Preparation of standard bacterial suspension

One milliliter aliquots of 24 h broth cultures of the standard organisms were aseptically distributed onto nutrient agar slopes and incubated at 37 ºC for 24 h. The bacterial growth was harvested and washed off with sterile normal saline, to produce a suspension containing about 108 - 109 colony forming units per mL The suspension was stored in refrigerator at 4 ºC until used (Kavanagh, 1972).

2.6. Preparation of standard fungal suspension

The fungal cultures were maintained on Sabouraut dextrose agar, incubated at 25ºC for three days. The fungal growth was harvested and washed with sterile normal saline, finally suspended in 100 mL of sterile normal saline, and the suspension was stored in the refrigerator until used (Kavanagh, 1972).

2.7. Antibacterial activity

Antibacterial activity was studied by an agar- diffusion method. Each of the incula of the tested organisms (1 mL) was poured into sterile Petri dish. A medium (at about 45ºC) was poured into each of the Petri dishes (20 mL). The medium was left to stand to allow it to set. Holes (disc shape) were made on the media with the aid of a sterile cork borer of 10mm diameter and removal of the agar disc. The cups were marked, and different concentrations of the sterile extract were pipetted (0.1 mL) into the cups using sterile syringes. Plates were then incubated at 37 ºC for 24 h. The sensitivities of the test organisms to the extracts were indicated by clear zones of growth inhibition around the cups containing the extracts and the diameter of the clear zone was taken as an index of the degree of sensitivity (Hassan *et al*., 2010).

2.8. Antifungal activity

Antifungal activity was studied by an agar- diffusion method. Each of the incula of the tested organisms (1 mL) was poured into sterile Petri dish. A medium (about 45ºC) was poured into each of the Petri dishes (20 mL). The medium was left to stand to allow it to set. Holes (disc shape) were made on the media with the aid of a sterile cork borer of 10mm diameter and removal of the agar disc. The cups were marked, and different concentrations of the plant extracts were pipetted (0.1 mL) into the cups using sterile syringes. Plates were then incubated at 37ºC for 48 h. The sensitivities of the test organisms to the plant extracts were indicated by clear zones of growth inhibition around the cups containing the plant extracts and the diameter of the clear zone was taken as an index of the degree of sensitivity (Hassan *et al*., 2010).

2.9. Phytochemical screening tests

Phytochemical screening was done for black mahlab seeds samples by using methods described by Sofowora (1993) and phytochemical analysis results were reported in Table 5.

2.10. Phytochemical screening

Ten gram of the powdered seeds was macerated in 100 mL of 70% (v/v) ethanol for 4h. The solution was filtered; enough 70% (v/v) ethanol added to complete the volume to 100mL. This prepared extract (PE) was used for the various phytochemical tests.

2.10.1. Test for flavonoids. 17.5 mL of the PE was evaporated to dryness on a water bath, cooled, and the residue was defatted by several extractions with petroleum ether. The defatted residue was dissolved in 30mL of 80% (v/v) ethanol, filtered, and the filtrate used for the following tests:

2.10.1.1. To 3 mL of the filtrate in a test tube, 1 mL of 1% of aluminum chloride in methanol solution was added. Formation of a yellow color immediately was taken as evidence of presence of flavonoids.

2.10.1.2. To 3 mL of the filtrate in a test tube, 1 mL of 1% potassium hydroxide solution was added. A dark yellow color indicates the presence of flavonoid.

2.10.2. Test for tannins. 7 mL of the PE was evaporated to dryness on a water bath. The residue was extracted several times with *n*- hexane and filtered. The insoluble residue was stirred with 10 mL of hot saline solution. The mixture was cooled, filtered, and the volume of filtrate adjusted to 10 mL normal saline.

2.10.2.1. Five milliliter portion of this solution was treated with few drops of gelatin salts reagent. Formation of immediate precipitate was taken as evidence for the presence of tannins in the sample.

2.10.2.2. To another portion of this solution, few drops of ferric chloride (test reagent) were added. Formation of blue, black or green color was taken as evidence for the presence of tannins in the sample.

2.10.3. Test for triterpens and unsaturated sterols. Ten milliliter of PE was evaporated to dryness on a water bath and the cooled residue stirred several times with

chloroform to remove the most of coloring materials. The residue was then extracted with 20 mL of chloroform. The chloroform solution was dehydrated over anhydrous sodium sulphate. Five mL portion of the chloroform solution was mixed with 0.5 mL of acetic anhydride followed by 2drops of conc. sulfuric acid. The gradual appearance of green, blue, pink to purple color was taken as evidence of presence of sterols (green to purple) and triterpens (pink to purple) in the sample.

2.10.4. Test for alkaloids. 7.5 milliliter sample of the prepared extract was evaporated to dryness on water bath. 5 mL 2N hydrochloric acid was added, stirred while heating on a water bath for 10min, cooled, and filtered. To test tube few drops of Mayer's reagent was added. Slightly turbidity or heavy precipitate was taken as evidence for the presence of alkaloids.

2.10.5. Test for saponins. One gram of the dried powdered seed was placed in a clean test tube. 10 mL of distilled water was added to test tube, stoppered, and vigorously shaken for about 30 s. The tube was then allowed to stand; the appearance of honey comb, persisted for at least an hour, was taken as evidence for the presence of saponins.

2.10.6. Test for cyanogenic glycosides. Three gram of the powdered seed was placed in flask and sufficient water added to moisten the sample, and followed by 1mL of chloroform. Apices of sodium picrate paper were carefully inserted between a split cork which used to stopper the flask. A change in color of the paper from yellow to various shaded red was taken as evidence for the presence of cyanogenic glycosides.

2.10.7. Test for anthraquinones. Ten gram of the powdered seed was boiled with 10 mL of 0.5 N KOH containing 1mL of 3% of hydrogen peroxide solution. The mixture was extracted by shaking with 10 mL of benzene. Five milliliter of the benzene solution was shaken with 3 mL of 10% ammonium hydroxide solution and the two layers allowed separating. The presence of anthraquinone was indicated by alkaline layer was found to have assumed pink or red color.

2.10.8. Test for coumarins. Three gram of the original powdered plant material boiled with 20 mL of distilled water in test tube and filter paper attached to the test tube. 0.5N KOH was added, and then the filter paper inspected under UV light.

3. Results and Discussion

Extensive experimental work was done to select the most suitable extraction methods (maceration, infusion, decoction and Soxhlet) for antimicrobial activity tests against Gram positive bacteria, Gram negative bacteria and fungi, particularly those cause respiratory tract infections.

Maceration by water resulted in an extract with many limitations and drawbacks, as the high susceptibility to fermentation that took place after 24 h. It gave positive results against *Staphylococcus aureus* and *Klebsiella pneumoniae* as illustrated in Table 2.

Maceration by different concentrations of methanol and ethanol in water was found to be the best extraction method. Ethanol 70% (v/v) was selected for antimicrobial test, due to its high extraction yield (Table 1), and for economic factors (ethanol has low cost, more available and safer than methanol). Ethanol 70% (v/v) gave positive results against Gram negative bacterium *Klebsiella pneumoniae* and Gram positive bacterium *Staphylococcus aureus* (Table 2). In infusion method, the hot infusion gave high extraction yield than cold one (Table1). This method (hot infusion) did not give results as shown in Table (2). In decoction method, decoction for 20min gave high extraction yield than others (Table 1). It did not give positive results as shown in Table (2). In Soxhlet method, the extract by methanol was superior to its high extraction yield (Table 1). Soxhlet extract by methanol did not give results as shown in Table 2.

The negative antimicrobial results showed by infusion, decoction and Soxhlet extracts Table 2, may be attributed to thermolabile natures of active compounds of extract which decomposed by temperature more than 40 ºC.

Pseudomonas aeruginosa and all fungi used were found to be insensitive to all extracts used as shown in Table 2. The resistance of *Pseudomonas aeruginosa* to extracts may be due to its permeability barrier afforded by its outer membrane lippolysaccharide and its tendency to colonize surfaces in the biofilm form makes the cells impervious to therapeutic concentrations of antibiotics (Omolola, 2011). The struggle of all fungi used in experiment to extracts may be due to its unique cell wall components glucans and chitin, make the diffusion of the extract difficult.

The phytochemical screening test results of seeds of *M. ciliatum* (Table 5), illustrated the presence of flavonoids, tannins and terpenoids which their anti-

allergic, anti- inflammatory, and antibacterial activities supporting the traditional uses of seeds for cold and allergic conditions. Medicinal plants have been used for centuries in folk medicines as remedies for human diseases, and many studies have been carried out in order to find out the scientific basis for their effectiveness. The main active antimicrobial agents isolated include alkaloids, phenolic acids, quinones, tannins, coumarins, flavonoids, and terpenoids (Ahmed *et al*., 2006).

M. ciliatum seeds in this study exerted a high antibacterial activity against Gram positive bacteria *Staphylococcus aureus* and Gram negative bacteria *Klebsiella pneumoniae*. The study, therefore, provides the scientific basis for its traditional application as ethno-medicine for cold and allergic conditions. Therefore, this medicinal herb can be used for the treatment of respiratory tract infections caused by these two bacteria. The whole plant extract was chosen for the test due to the probability of synergistic action.

Table 1. The yield percentage and some organoleptic properties of extract

Extraction method	Yield % (± 0.5 %)	Color	Odor	Taste	Consistency
Maceration by water	9.05	yellow	aromatic	bitter	solid
Maceration by methanol 30%	5.15	brown	aromatic	bitter	semisolid
Maceration by methanol 50%	5.45	brown	aromatic	bitter	semisolid
Maceration by methanol 70%	7.40	brown	aromatic	bitter	semisolid
Maceration by ethanol 30%	2.45	brown	aromatic	bitter	semisolid
Maceration by ethanol 50%	5.00	brown	aromatic	bitter	semisolid
Maceration by ethanol 70%	5.15	brown	aromatic	bitter	semisolid
Infusion by cold water	6.50	yellow	aromatic	bitter	solid
Infusion by hot water	9.50	brown	aromatic	bitter	solid
Decoction by water 5min	7.15	brown	aromatic	bitter	solid
Decoction by water 10min	12.13	brown	aromatic	bitter	solid
Decoction by water 15min	12.60	brown	aromatic	bitter	solid
Decoction by water 20min	13.05	brown	aromatic	bitter	solid
Soxhlet by methanol	9.35	brown	aromatic	bitter	semisolid
Soxhlet by ethanol	6.00	brown	aromatic	bitter	semisolid
Soxhlet by chloroform	3.5	white	odorless	tasteless	liquid

Table 2. Comparative bioactivity of *Monechma ciliatum* seed extract against different standard bacteria and fungi

Extraction method	Solvent system	`Conc. (mg\mL)	Standard bacteria			Standard fungi		
			Sa	Pa	Kp	An	Ca	Af
Maceration (24 h)	water	25	11	-	-	-	-	-
		50	22	-	-	-	-	-
		100	26	-	-	-	-	-
		150	-	-	20	-	-	-
Maceration (24 h)	Ethanol (70%)	25	-	-	-	-	-	-
		50	11	-	16	-	-	-
		100	17	-	25	-	-	-
		150	11	-	33	-	-	-
Infusion (20 min)	water	25	-	-	-	-	-	-
		50	-	-	-	-	-	-
		100	-	-	-	-	-	-
		150	-	-	-	-	-	-
Soxhlet (10 h)	methanol (70%)	25	-	-	-	-	-	-
		50	-	-	-	-	-	-
		100	-	-	-	-	-	-
		150	-	-	-	-	-	-

Data are presented as mean diameter of growth inhibition zone (mm), average of three replicates, inhibition zone 15 mm: sensitive, 14-15 mm: intermediate, < 15 mm: resistant, - : no inhibition zone.

Table 3. Antibacterial activity of ampicillin and gentamicin

Drug	Conc. (mg/ml)	MDIZ(mm) Standard bacteria used		
		Sa	Pa	Kp
Ampicillin	0.04	30	-	-
	0.02	26	-	-
	0.01	22	-	-
	0.005	14	-	-
Gentamicin	0.04	18	22	16
	0.02	17	15	14
	0.01	14	12	-
	0.005	13	-	-

MDIZ: mean diameter of growth inhibition zone (mm), average of two replicates, inhibition zone ≥ 15 mm: sensitive, 14-15 mm: intermediate, < 15 mm: resistant, -: no inhibition zone

Table 4. Antifungal activity of nystatin and clotrimazole

Drug	Conc. (mg/ml)	MDIZ(mm) Standard fungi used		
		Ca	An	Af
Nystatin	0.05	28	17	-
	0.025	26	14	-
	0.0125	23	-	-
Clotrimazle	0.02	34	24	-
	0.01	30	29	-
	0.005	33	17	-

MDIZ: mean diameter of growth inhibition zone (mm), average of two replicates, inhibition zone ≥15mm :sensitive, 14-15mm: intermediate, < 15mm: resistant, -:no inhibition zone.

Table 5. The general phytochemical test results of *Monechma ciliatum* seeds

No.	Test	Result
1	Alkaloids	-ve
2	Anthraquinones	+ve
3	Coumarins	-ve
4	Cyanogenic glycosides	-ve
5	Flavonoids	+ve
6	Saponins	-ve
7	Tannins	+ve
8	Triterpenes and unsaturated sterols	+ve

As phytomedicines gain popularity, it is essential to educate the medical and scientific establishment that the pharmacological model of isolating constituents may not be the best methodology for the study of herbal medicines. The potentiation (or antagonism) provided by perturbation of multiple pharmacological targets converging in parallel biochemical pathways is often missed by standard laboratory searches for activity. If a chemical matrix is necessary for activity in phytomedicines, then many remedies from the purification process from whole plant to isolated constituent may be overlooked. Ultimately, effective research into the mechanisms of actions of phytomedicines will need to account for the possibility, indeed the probability, of synergistic activity between multiple constituents (Cseke *et al*., 2006).

4. Conclusion

The high antibacterial activity of *Monechma ciliatum* seeds can be used to source antibiotic substances for drug development that can be used in the control of respiratory tract bacterial infections. Further investigations of its activity against a wider range of bacteria and fungi, identification and purification of its chemical constituents, and toxicological investigations of the plant extracts should be carried out with a view to developing novel drugs for human consumption.

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