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The Titer Testing in Post-Vaccination Rabies Immunoglobulin G (IgG) with the Administration of Wild Horse Milk

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Abstract

The occurrence of human rabies in Indonesia approximately amounted to 168 cases. The countermeasures taken were vaccination, quick treatment for individuals, KIE (*Komunikasi, Informasi, dan Edukasi*; Communication, Information, and Education), surveillance, selective dog elimination and post-exposure management. Vaccination was one of the effective countermeasures against the distribution of rabies. It might be conducted either before or after the virus exposure. Despite its effectiveness, it could end in failure due to several factors, one of which was the nutritional status of patient. However, the failure could be overcome by enhancing the immune system (immunostimulator) with the administration of wild horse milk. The milk contained lactoferrin known as protein inducing antibody. Sample consisted of 15 rabbits. They were divided into three groups. Each group consisted of five rabbits. Group t1 for titer testing in immunoglobulin after anti-rabies vaccination; while Group t2 and t0 for titer testing in immunoglobulin after rabies-vaccination and administration of wild horse milk and for the control group without any treatment respectively. The results showed a titer increase in Ig G after vaccination by 40% in Group t2 and t2 ($p>0.05$). Besides, the administration of wild horse milk could increase titer in IgG after vaccination for two weeks ($p<0.05$). In Conclusion, Administration of wild horse milk could increase titer in IgG after vaccination.

1. Introduction

Rabies is caused by the rabies virus, a virus species of *Lyssavirus* genus in the family *Rhabdoviridae*. The neurotropic virus can develop in the nervous tissue. Human can be infected by the virus if bitten by rabid dogs, cats, monkeys or bats **(Johnson, Cunningham and Fooks, 2010; Shi *et al.*, 2018)**. Rabies has spread all over the continents and annually caused 59,000 deaths in over 150 countries. There were 95% of rabies cases in both Africa and Asia **(World Health Organization, 2018)**.
The annual occurrence of human rabies in Asia was as follows: 20,000 cases in India; 2,500 cases in China; 20,000 cases in the Philippines; 9,000 cases in Vietnam and 168 cases in Indonesia. 24 of 34 provinces in Indonesia was endemic; while the ten others were considered rabies-free. According to the data of GHPR (Animal Transmitter Rabies Bite) cases issued by the Ministry of Health, GHPR had increased by 86.3; from 45,466 (2009) to 84,750 cases (2012). The increase was due to KLB (Extraordinary Condition) in Bali in 2009-2012.
24 of 34 provinces in Indonesia was endemic; while the ten others were considered rabies-free. According to the data of GHPR (Animal Transmitter Rabies Bite) cases issued by the Ministry of Health, GHPR had increased by 86.3; from 45.466 (2009) to 84.750 cases (2012). The increase was due to KLB (Extraordinary Condition) in Bali in 2009-2012 **(Indonesian Health Ministry, 2014; Agustina *et al.*, 2018)**.
Furthermore, the KLB was a trending topic again in 2019. Rabies endemically occurred in West Nusa Tenggara, specifically in Dompu and Sumbawa. The first occurrence was in Dompu then spread to Sumbawa. According to the findings of epidemiologic investigation by an integrated team from Ditjen P2P Ministry of Health and Ditjen PKH Ministry of Agriculture, there were 192 cases of rabid animal bite and two death cases in human bitten by rabid animals. Meanwhile, according to weekly reports from Health Office and Livestock Service of Dompu, there were 735 cases of rabid animal bite and six death cases in human bitten by rabid animals until the third week in February 2019 **(Indonesian Health Ministry, 2014; Nadine-Davis, 2015; Agustina *et al.*, 2018)**.
Human can be infected by rabies through various ways, particularly when their exposed skin or mucous membrane having contacted with blood contaminated by saliva of individuals bitten by rabid animals **(Zhu *et al.*, 2015)**. Ministry of Health, with the Governments of Dompu and Sumbawa collaborated to overcome KLB of rabies in West Nusa Tenggara. The countermeasures were vaccination, quick treatment for individuals bitten by rabid animals (washing the bitten area using a soap and running water for 15 minutes), KIE, surveillance, selective dog elimination, and post-exposure management for human **(Reni *et al.*, 2010; Agustina *et al.*, 2018)**.

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Vaccination is an effective countermeasure against the spread of rabies. It may be administered either before or after the virus exposure. Rabies vaccine contains inactive virus derived from the continuous cell pathway. The virus is administered in intramuscular or intradermal way in accordance with the protocol recommended by WHO. Recommended pre-exposure vaccination should be administered to health workers, laboratory workers and travelers in endemic areas. Moreover, post-exposure treatment or post-exposure prophylaxis (PEP) is an effective treatment against rabies. PEP contains of vaccination and rabies immunoglobin (RIG) effective to prevent diseases. A successful rabies vaccination indicates antigen and antibody titer formed by a certain product of vaccine induced after inoculation **(Faisal *et* al., 2010; WHO, 2018).**

One of the efforts to minimize any failure in post-exposure vaccination is to administer substances that may increase body immune response, or immunostimulator. We can use healthy food products derived from either plants or animals. For example, milk, having an active component can be regarded healthy due to its nutrition content that prevents and cures diarrhea, impaired mineral adsorption and immunodeficiency. Furthermore, protein in Sumbawa horse milk contains lactoferrin, lactoperoxidase, lysosome and immunoglobin known as antimicrobial protein. Several research found that Sumbawa wild horse milk can serve as an immunomodulator. The milk contains lactobacillus and destroys bacteria cells, enhances immune responses (increasing the IgA and IgG production) and activates macrophage and specific antibody response against foreign antigens **(Faisal *et al.*, 2010; Reni *et al.*, 2010)**.

The administration of Sumbawa wild horse milk, according to some research *in vivo*, can enhance immunity in Hepatitis A vaccine given to Balb/c mice. It indicates that the milk can increase receptor capacity of macrophage and cytokine production that activates macrophage. In addition, the milk contains lactoferrin that induces the formation of antibody **(Yuki, 1998; Nadine-Davis, 2015)**. The research aims to analyze titer in IgG after the post-vaccination administration of wild horse milk. It also aims to investigate the variation of immune response given by experimental animals administered with anti-rabies vaccine.

2. Material and methods

2.1 Treatments in Experimental Animals

The experimental animals were healthy male rabbits aged seven-eight weeks old (weight of 700-800g). The research had been approved by the Ethic Commission of Faculty of Medicine, Universitas Mataram (Approval Number: 251/UN18.F7/ETIK/2019).

The rabbits were divided into three groups i.e. rabbits without treatment (t0), rabbits with vaccination (t1) and rabbits with vaccination and wild horse milk (t2). The research was performed in six treatment stages i.e.:

Stage 1: acclimatization process in rabbits adjusting to their food, water and laboratory condition for seven days.

Stage 2 : blood sample taking in rabbits before anti-rabies vaccination

Stage 3 : administration of anti-rabies vaccination in the treatment group

Stage 4 : blood sample taking in rabbits after anti-rabies vaccination

Stage 5 : administration of wild horse milk in the group administered with rabies vaccine

Stage 6 : final blood sample taking after the administration of rabies vaccine and wild horse milk

Sample blood taken in each treatment stage was investigated. We analyzed the titer in IgG using ELISA method.

2.2 Anti-rabies Vaccination and Administration of Wild Horse Milk

PVRV (Purified Vero Rabies Vaccine) consisted of dried vaccine in a vial and solvent of 0.5 mL in a syringe. Rabbits in the treatment groups were administered with PVRV of 0.5mL in an intraperitoneal way. Two weeks later, they were administered with wild horse milk. Meanwhile, rabbits in the control group were administered with aquadest and usual rabbit food. Wild horse milk was directly administered. We filled a drink container with the milk in such a way, preventing it to spill out. For the administration, the rabbits were individually caged. It eased our evaluation. They were administered milk of 300 mL/day/rabbit. We took the rabbits' blood through their cubital vain following the administration. The blood sample was frozen for two hours. It was centrifuged at 3000 rpm for ten minutes. The serum was separated for our investigation object. The bled rabbits were killed and buried to prevent the spread of rabies.

2.3 Statistical Analysis

The effect of the administration of wild horse milk on rabbits was analyzed using One Way Anova. The difference between two treatment groups was analyzed using Paired t-test ($p<0.05$).

3. Results

Rabies vaccine was administered to the rabbits in a subcutaneous way. Table 1 shows the result of titer testing in IgG two weeks before and after the administration.

Table 1. Titer Testing in IgG Before and After Anti-rabies Vaccination

Treatment Group	Titer in IgG (IU/mL)		P
	Before Vaccination	After Vaccination	
t0	0.26 ± 0.08	0.24 ± 0.05	0.374^{ns}
t1	0.22 ± 0.04	0.88 ± 0.79	0.128^{ns}
t2	0.20 ± 0.07	0.84 ± 0.89	0.127^{ns}
p	0.420^{ns}	0.279^{ns}	

Data were in forms of mean value and standard deviation.
p : significance value with a 95% confidence level
t0 : normal control group without any administration of vaccine and milk
t1 : vaccinated group
t2 : vaccinated group administered with milk

Table 2. The Result of Titer Testing in IgG After the Administration of Vaccine and Wild Horse Milk

Treatment Group	Titer in IgG (IU/mL)		P
	After Vaccination	Post-vaccination with Milk Administration	
t0	0.24 ± 0.05	0.20 ± 0.07	0.374^{ns}
t1	0.88 ± 0.79	0.90 ± 0.77	0.374^{ns}
t2	0.84 ± 0.89	1.24 ± 1.07	0.037^{*}
P	0.279^{ns}	0.134^{ns}	

Data were in forms of mean value and standard deviation
p : significance value with a 95% confidence level
t0 : normal control group without any administration of vaccine and milk
t1 : vaccinated group
t2 : vaccinated group administered with milk

Table 1 indicates an insignificantly increased titer in immunoglobin G (IgG) ($p>0.05$). The mean of titer in IgG after

vaccination <1.0 IU/mL indicated a negative zero. Group t2 was administered wild horse milk for two weeks. The post-vaccination titer in their IgG was then analyzed.
Group t2 indicated a significantly increased titer in IgG ($p = 0.037$); while Group t0 and t1 did not ($p>0.05$). The mean of titer in IgG after the administration of vaccine and wild horse milk 1.24 IU/mL indicated a positive zero.

4. Discussion

Rabies is a zoonosis disease due to RNA virus from the genus Lyssavirus, the family *Rhabdoviridae*. It attacks the central nervous system of both human and mammals. The primary reservoir of rabies is domestic dogs. Most cases (98%) were triggered by dogs' bite; while other cases were by monkeys and cats'. Unvaccinated patients may lead to death by 100% **(Krebs *et al*., 2003; Johnson *et al*., 2010)**. We used uninfected rabbits in this research. We vaccinated them and gave them milk after the vaccination. We intended to analyze the titer in their IgG based on the capability of the rabies vaccine used and the administration of wild horse milk.
Administration of rabies vaccine aimed to develop the active immune system of rabid patients through the humeral immune system and specific immune system. The first system manifested an antibody that would neutralize viruses existing outside the cell; while the later manifested CTL that would destroy rabid cells. Activation of humeral immune response was started by phagocytizing viruses by antigen presenting cells (APC). Following the process, antigens would be presented to helper T lymphocytes. The cells produced various mediators that would activate cell B to be plasma cells producing antibody. Besides, the mediators also activate other T cell sub-sets to be specific cytotoxic cells **(Aubert, 1992; Salimei and Fantuz, 2013)**. Referring to the findings, there was a titer increase in immunoglobulin G (IgG) two weeks after vaccination. However, statistically speaking, there was no significantly different titer increase before and after vaccination. Such difference was triggered by various factors; such as immune responses to vaccination process emerging seven-ten days after vaccination. The response might lead to the peak on the 28th day after vaccination **(Togawa *et al*., 2002; Salimei and Fantuz, 2013)**. Another factor was that animal condition. The animal condition would give different response to the vaccine. Furthermore, vaccination route, animal's age while being vaccinated, type of vaccine, vaccination schedule and animal's origin and health status were other factors. Unvaccinated normal group indicated a negative zero in all rabbits due to the absence of immune response **(Johnson *et al*., 2010; Shi *et al*., 2018).**
We also figured out that the administration of wild horse milk could increase titer in IgG of group t2 after vaccination significantly ($p<0.05$). The milk served as an immunomodulator due to its lactoferrin glycoprotein important for body immune system. Glycan bound in the milk lactoferrin was complex. We revealed the complexity after checking it in an immunoblotting way by Con-A and WGA lectin labelled using peroxidase. Con-A lectin was specifically bound to high-mannose glycan. Horse milk lactoferrin had a complex glycan. As a result, the lactoferrin could increase immune response. It stimulated the activity of peritoneal macrophage phagocytosis and increased cells producing IgA in the intestinal tissue **(Hurley *et al*., 1993; Kuwata *et al*., 2001; Togawa *et al*., 2002)**. Glycan in horse milk lactoferrin was similar to that in breast milk lactoferrin. The components of the glycan gave implications to the increase in brush border membrane. Due to fragment adsorption, lactoferrin in intestinal cells could pass the non-specific interaction with glycosaminoglycan and be specifically interacted through receptor **(Debbabi *et al*., 1998; Faisal *et al*., 2010; Blaise and Gautret, 2015)**.
Increased titer in IgG after the administration of vaccine and wild horse milk was also caused by protein in milk. The protein underwent an autofermentation process, increasing humeral immune responses. The increase activated specific immune responses. β-lactoglobulin and α-lactoalbumin in milk and lactoferrin served as an antivirus inhibiting virus replication through a special signal in virus cycles **(Bojsen *et al*., 2007; Banyard and Fooks, 2011).** Lactic acid bacteria produced by wild horse milk served as a probiotic, a strong activator for immune response due to its specific molecules on the cell wall surface. The bacteria could affect lymphocytes for immunoglobulin secretion **(Togawa *et al*., 2002; Tang *et al*., 2005; Fotschki *et al*., 2016).**
The results of titer increase in antibody of rabbits administered wild horse milk after vaccination was in line with the findings of other research. The research argued that fermented horse milk increased IgA concentration in the serum by 46.20% and lymphocytes by 95.47%. The horse milk was administered to mice's inmmunoglobuline (IgA) antibody after hepatitis A vaccination **(Kuwata *et al*., 2001; Faisal *et al*., 2010)**. Horse milk could also increase the activity of macrophage in cellular immune in mice against salmonella **(Hurley *et al*., 1993; Togawa *et al*., 2002)**. Furthermore, perioral administration of fermented Sumbawa horse milk could increase the potential of Vibrio cholera conjugated with cholera toxin in term of significant S-IgA immune response induction. The milk protectively prevented liquid secretion in the intestines of Balb/C mice. It indicated that components of Sumbawa wild horse milk could increase the capacity of receptor in macrophages. In addition, the components could also increasingly produce cytokines activating macrophages. Besides, the milk lactoferrin could induce the formation of antibody **(Rupprecht *et al*., 2007; Reni *et al*., 2010; Fotschki *et al*., 2016)**.

5. Conclusion

Administration of wild horse milk could increase titer in IgG after anti-rabies vaccination in rabbits.

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Declaration of interest

The authors report no conflicts of interest.

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Isolation of Aerobic Bacteria Flora in the Gills and Gastrointestinal Tract of Culturable Freshwater Fish from Ogbia Bayelsa State

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Abstract
Fish is in high demand as food, food additives, and supplements as they are a rich source of carbon, proteins, vitamins, and minerals. Fish has been established to possess bacterial populations on or in their skin, gills, digestive tract, etc. with their microbial diversity often reflecting the bacterial populations of the surrounding water which are either allochthonous or allochthonous. This study isolated and enumerated aerobic bacteria flora in the gastrointestinal tract and gills of four culturable freshwater fish (Silver catfish, Tilapia, Clarias, and Heterobranchus). These species of cultural freshwater fish were obtained and each adult specie held in a separate glass containing unchlorinated water and transferred to the laboratory. The quantitative and qualitative estimation of the bacteria flora present in the gill and Gastro-Intestinal Tract (GIT) of fish were investigated. The Mean total plate count on Nutrient Agar (NA), Blood Agar (BA), MacConkey Agar (MCA), Cysteine-Lactose-Electrolyte-Deficient Agar, (CLED Agar) and Salmonella – Shigella Agar (SSA) was found to be 60 and 40 CFU, 150 and 80 CFU, 100 and 90 CFU and 80 and 70 CFU respectively. Most of the isolates were of public significance. The results showed that fish contains a large number of microbiotas which may play a role in nutrition and health.

1. Introduction

Fish is in high demand as food, food additives and supplements as they are a rich source of carbon, proteins, vitamins, and minerals. Fish has been established to possess bacterial populations on or in their skin, gills, digestive tract, and light-emitting organs, internal organs (kidney, liver, and spleen) with their microbial diversity often reflecting the bacterial populations of the surrounding water (Austin, 2002). These microbiotas are either allochthonous bacteria (normal flora) or allochthonous (opportunistic and transient) (**Ringo *et. al.*, 1995**). The composition of the allochthonous intestinal tract microbiota is highly variable and is affected by many environmental conditions as salinity, temperature, etc. (**Liu *et al.*, 2008; Pond *et al.*, 2006; Ringo *et al.*, 1995**), but stable in fish kept in defined conditions (Pond *et al.* 2006). Food accessibility, composition and changes may affect the bacterial diversity in a fish intestine (**Ringo & Strom, 1994**; **Ringo *et al.*, 2006**). The diversity of the microbiotas of the fish intestine has been shown to be largely dependent on the bacterial colonization during their early development (**Ringo & Birkbeck 1999; Ringo *et al.*, 1995)** and often reflect those of the surrounding water (**Austin, 2002**). However, some studies have also reported a wider diversity of the gut microflora than previously believed (**Ringo *et al.* 2006; Hovda *et al.* 2007; Ward *et al.* 2009**), especially in the intestinal contents of freshwater fish (**Cantas *et al.*, 2012; Gonzalez *et al.*, 1999; Spanggaard *et al.*, 2000; Wu *et al.* 2010**). This study is aimed at isolating and enumerating the aerobic bacteria flora from the gastrointestinal tract of culturable freshwater fish from a fish pond in Ogbia, Bayelsa State.

2. Material and methods

2.1 Sample Site

The samples were collected at Ogbia (4° 39′ 00″ N 6°16′00″ E), a Local Government Area of Bayelsa State in the Niger Delta region of Nigeria. It has an area of 695 km^2 and an estimated population of 179,926. It is headquartered to Oloibiri where crude oil was first discovered in Nigeria in 1956.

2.2 Sample Collection

The fish sample was collected with aquatic dip net into clean containers, appropriately labeled and taken to the laboratory for analysis.

2.3 Isolation of Microbes

Samples of silver cat fish, Tilapia, Clarias and Heterobratis were collected from a fish pond in Otuaba Community, Ogbia L.G.A of Bayelsa State. Each adult species of the fish was held in a separate glass containing unchlorinated water during the

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transfer to the laboratories. They were sacrificed by pithing. The ventral surface of the fish was carefully scrubbed with 1 % iodine solution for surface decontamination (**Trust & Sparno, 1974**) and dissected under aseptic conditions. The gill portion and GIT portion were homogenized individually with distill water and 1 ml of the sample plated in triplicate on nutrient agar for evaluation of the total plate count, Salmonella – shigella agar for total salmonella shigella counts, MacConkey agar for total coliform count and blood agar (as a selective media) for streptococcus and staphylococcus count). The Plates were incubated at 37 ^{o}C for 24 hours aerobically to count bacteria colonies. The distinct colonies (based on their different morphological, character (color, colony, size, surface, margin and opacity), were sub cultured on the respective media to obtain pure culture.

2.4 Identification and Characterization of Microbes

Phenotypic identification of microbes was performed according to standard methods (**Barrow and Feltham, 2003**). Expressed microbial morphological traits examined include the orientation, size, and pigmentation which were performed by visual inspection of microbial isolates on petri-plates, as well as cell wall characteristics which was performed by Gram staining of the isolates. Expressed biochemical traits examined include: the production of coagulase enzyme (coagulase test); the production of catalase enzyme (catalase test); the production of urease enzyme (urease test); biodegradation of tryptophan to produce indole (indole test); utilization of citrate as a sole carbon source (citrate test); production of stable acids from glucose fermentation (methyl red test); production of acetoin as the main end product with small quantities of mixed acids from glucose metabolism (Voges Proskauer test); and motility.

3. Results

Table 1. The Prevalence of Aerobic Bacteria in GIT and Gills of Culturable Fresh Water Fish (CFU)

	No of isolates	***Escherichia* coli**	***Staphylococcus* aureus**	***Proteus* sp**	**Pseudomonas sp**	***Salmonella* sp**	***Vibrio* sp**	***Klebsiella* sp**
Silver Catfish								
GIT	60	10	5	-	10	5	20	10
Gill	40	3	9	-	2	8	11	7
Tilapia								
GIT	150	40	25	10	20	6	30	19
Gill	80	15	5	-	17	21	22	-
Clarias								
GIT	80	5	-	-	10	20	25	20
Gill	70	-	-	23	18	14	15	-
Heterobratis								
GIT	100	10	30	24	20	-	16	-
Gill	90	6	10	-	-	18	31	25
	670	89 (13.28%)	84 (12.33%)	57 (8.51%)	97 (14.48%)	92 (13.73%)	70 (25.37%)	81 (12.80%)

Table 1 above showed that *E. coli, staphylococcus aureus, proteus* sp, *pseudomonas* sp, *salmonella* sp, *Vibrio sp*, *klebsiella sp* were the bacteria isolated. Vibrio had the highest occurrence in the GIT and gill of the fish samples (28.6%) while Proteus (8.92%) had the least occurrence of bacteria.

Table 2. Gram Negative and Positive Organisms Present

Test	**Probable Organism**						
	E. coli	***S. aureus***	***Proteus* sp.**	***Pseudomonas* sp.**	***Salmonella* sp.**	***Vibrio* sp.**	***Klebsiella* sp.**
Oxidase test	-	-	-	+	-	+	-
Catalase test	+	+	+	+	+	-	+
Coagulase test	-	+		-		-	
Indole	+	-	-	-	-	+	-
Methyl red test	+	+	+		+	-	-
Voges-Proskauer reaction	-	+	-		-		+
Urease	-	+	+	-	-	-	+
Citrate utilization	-	+	+	+	-	+	+
Motility	+	-	+	+	+	+	-
Gram staining	-	+	-	-	-	-	-

Table 3. Appearance of the Isolated Organisms on a Cultured Plate

Media	Appearance	Probable Organism
MacConkey Cled agar Blood agar	Smooth, glossy, translucent, rose pink colonies Smooth, circular, 1.5 mm diameter, yellow opaque colonies Colonies surrounded by zone of haemolysis	*Escherichia coli*
Blood agar	Large, round, golden-yellow colonies, with haemolysins	*Staphylococcus aureus*
Nutrient agar MacConkey agar Blood agar	Moist, translucent, round disks (1-2 mm in diameter) with a bluish tiny in transmitted light colonies Colonies became reddish on prolonged incubations The greenish zone initially appeared around the colonies and later became clear due to haemodigestion	*Vibrio* sp.
MacConkey agar	Mucoid red colonies with fishy smell	*Proteus* sp.
MacConkey agar	undulated white translucent, mucoid colonies	*Klebsiella* sp.
Cled agar	Heavily dull surface and irregular lines appeared with bluish green colour pigment	*Pseudomonas* sp.
MacConkey agar Blood Agar Salmonella – shigella	Non lactose fermenting, smooth, and pale colonies Non-hemolytic smooth white colonies Non fermenting colonies with black center	*Salmonella* sp.

4. Discussion

Fish living in a natural environment are known to harbor some pathogenic Enterobacteriaceae (**Pillay, 1990**). In this study seven bacteria viz *Escherichia coli*, *Staphylococcus aureus*, *Proteus* sp., *Pseudomonas* sp., *Salmonella* sp., *Vibrio* sp. and *Klebsiella* sp. were isolated. According to **Guzman *et al.* (2004),** the invasion of fish muscles due to breakage of immunological barrier of fish by pathogens is likely to occur when the fish are raised in pond with faecal coliforms such as *vibrio cholera, E. coli, S. aureus* etc. with greater than 10^4 – 10^{14} per 100 ml in pond water respectively. These bacteria isolated are all of public health significance and thus require close attention. Two of the isolates (Staphylococcus and Salmonella) are amongst the four most common types of food poisoning bacteria. The other two being clostridium and campylobacter. However, the other isolates apart from *Pseudomonas* have been frequently associated to food borne infections (**CDC, 2019; Wang *et al.*, 2010**). The interesting thing about some of these organisms like Staphylococci produce heat stable toxin that is not destroyed by cooking. The ingestion of contaminated fish or fish products that is not properly handled or cooked contributes significantly to cases of food borne illnesses. There is therefore a need to develop or adopt safe management practices for the production of fish or its product for human consumption (**Teophilo *et al.*, 2002**).

5. Conclusion

Fish is in high demand as either food, food additives or supplements. This study aimed at isolating and enumerating the aerobic bacteria flora from the gastrointestinal tract of culturable freshwater fish has demonstrated that the gills and guts of fresh water fish are a potential source of microorganisms of public health importance. If not properly prepared, consuming fresh fish form contaminated water can cause food borne diseases (poisoning and intoxication). Since there is a strong correlation between environmental contamination and the diversity of microbiome isolated from fish, it is vital that the proper environmental and public heath attention and commitment be given to the fish habitats. It is also pertinent that there is an increased awareness of proper preparation of these fishes before consumption.

Declaration of interest

The authors report no conflicts of interest.

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Effect of *Cercospora piaropi* Tharp and *Myrothecium roridum* Tode Fries Formulated as Corn Oil Emulsion on Water Hyacinth Shoot Growth under Greenhouse Conditions

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Abstract

A study was done to find out the comparative effect of *Cercospora piaropi* Tharp and *Myrothecium roridum* Tode Fries formulated as corn oil emulsion on water hyacinth shoot growth and biomass under greenhouse conditions. The study site was located in Kibos at latitude $0^0$37'S and longitude $37^0$20'E with average temperature of 25 to 30^0C and 22 to 27^0C during the day and night respectively, and 60 to 69% relative humidity. Healthy water hyacinth plants were inoculated with the pathogens formulated in corn oil at $1x10^9$, $1x10^8$, $1x10^7$, $1x10^6$ and $1x10^5$spores/ml. The control plants were not inoculated. The experiment was set up in completely randomized design (CRD) with each treatment replicated three times. At weeks 2, 4, and 6 after inoculation, the average shoot length and biomass for the treated basins were separately compared to the average shoot length and biomass of the control plants. Increase in spore density for both pathogens significantly increased relative shoot length and relative biomass. Relative shoot length was 55.07 and 51.93 for *C. piaropi* and *M. roridum* respectively at $1x10^9$ spores/ml while relative biomass was 73.53 for *C. piaropi* and 37.60 at $1x10^9$ spores/ml. Inoculation suppressed shoot elongation and biomass with $1x10^9$ spores/ml being most effective. *Cercospora piaropi* formulated in corn oil lowered shoot length and biomass of water hyacinth more than *M. roridum* did.

1. Introduction

Water hyacinth invasion and its associated effects to riparian communities poses challenges to activities like fishing and farming along invaded water bodies. Destruction of farm produce by flooding due to blocked drainage channels, increasing travel time used to access farms and consequent reduction in farmers income are some of the adverse effects of water hyacinth invasion **(Honla *et al.*, 2018)**. Though physical, chemical and biological control methods have been tried out, **VonBlank *et al.* (2018)** has stated that reoccurrence of the weed relies on biomass reintroduction by humans. The weed has therefore remained resurgent and difficult to manage **(Ongore *et al.*, 2018; Segbefia *et al.*, 2019)** courtesy of its high proliferation coupled with high seed production rate, ability for both sexual and asexual reproduction. High expenses have made physical control and herbicide application to be non-sustainable **(Worku and Sahile, 2018)**.
Much research on water hyacinth bio control has been devoted to the development of new mycoherbicide formulations using vegetable oil as the carrier material **(Berestetskiy and Sokornova, 2018)**. These formulations have not been effective due to reasons related to rapid water hyacinth luxuriant growth in terms of shoot growth and biomass accumulation with the added advantage of ecological adaptability **(Worku and Sahile, 2018)**. **Tobias *et al.* (2019)** reported that the weed growth in terms of stem elongation and biomass accumulation makes it have a propensity for compromising the economic use of the waterways. Management of shoot growth and biomass would open up the water for economic use **(Eid and Shaltout, 2017)**.
Studies have been carried out on using various vegetable oils from plants as formulation material for pathogens for water hyacinth control **(Boyette and Hoagland, 2013)**. While basically all pathogens interfere with primary plant defense, necrotrophs such as *Cercospora* and *Myrothecium* secrete toxins to kill plant tissue. Hence, *C. piaropi* and *M. roridum* isolates have potential for use in water hyacinth bio control. Cercosporin produced by *Cercospora* is able to lower the growth rate of water hyacinth **(To-Anun *et al.*, 2011)** while phytotoxins roridin A and roridin E produced by *Myrothecium* have been reported to be similar to paraquat and can be used for water hyacinth control **(Okunowo *et al.*, 2019)**. Generally, foliar pathogens working under natural disease pressure do not have the capacity to kill water hyacinth plants completely and quickly unless they can be used in conjunction with efficacy-enhancing formulations and adjuvants **(Charudattan, 2014; Mutebi *et al.*, 2013)**, a

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formulation being the form of a specific product that is used to control a pest **(Libs and Salim, 2017)**. Recent trends in the implementation of bio herbicide use in the control of water hyacinth have depended primarily on several strategies **(Okunowo *et al*., 2019)**. The use of bio formulations has been stimulated as part of the search for alternatives to chemical control, as the use of environmentally friendly formulations minimizes hazards resulting from herbicide residues **(Dagno *et al*., 2012)**. Inert solid carriers, alginate granules, invert emulsions and oil-in-water emulsions have been considered as vehicles for mycoherbicides as they reduce or eliminate the dew requirement for fungal colonization **(Berestetskiy and Sokornova, 2018)**. A commonly used formulation material has been corn oil, a vegetable oil that is gotten mostly by aqueous extraction methods from maize germ **(Shende and Sidhu, 2014)**. It is generally less expensive than most other types of vegetable oils, harmless to the environment, highly biodegradable and used domestically in foods **(Kaltragadda *et al*., 2010)**. A quality that qualifies corn oil as a formulation agent is its low viscosity that makes dispersal of spores within the oil easy during spore harvesting and formulation **(Boyette and Hoagland, 2013)**.
The purpose of this study was to compare effect of C*ercospora piaropi* Tharp and *Myrothecium roridum* Tode Fries formulated as corn oil emulsion on water hyacinth shoot growth under greenhouse and make a choice of the pathogen between them that can be used as corn oil formulation for the control of water hyacinth.

2. Material and methods

The study was carried out in a greenhouse at Kibos in Kisumu situated at latitude 0⁰37'S and longitude 37⁰20'E. It is about 10 km from Lake Victoria. Temperature averages were 25 to 30⁰C and 22 to 27⁰C during the day and night respectively while the relative humidity averages varied from 60 to 69%.
Cercospora piaropi and *Myrothecium roridum* were isolated from infected plants and aseptically cultured, sub cultured and spores harvested following procedure by **Groenewald *et al.* (2013)** and of **Kwon *et al*. (2014)** for the two pathogens respectively.
Following the method of **Tahlan (2014)**, 100 mls of refined domestic grade corn oil obtained from a local shopping mall was measured and put into a sterilized cone flask and topped up to 1000 mls (1 liter) with sterilized distilled water. One milliliter of 1% polysorbate was added to the contents of the cone flask and the mixture thoroughly shaken to form a 10% corn oil emulsion. After the surface of *C. piaropi* turned red and *M. roridum* turned dark indicating sporulation for the two pathogens, the corn oil emulsion was repeatedly pipetted over the surface of each of the cultures until the emulsion in the pipettes became cloudy. The contents of the pipettes were then separately plunged into sterilized beakers as *C. piaropi* and *M. roridum* stock solutions. The solutions were refrigerated at 5⁰C awaiting usage. A haemocytometer was used to determine the concentration of the spores in the suspension employing the method created by **Caprette (2000)**. The concentration of the stock solution was adjusted and by serial dilution to $1x10^9$, $1x10^8$, $1x10^7$, $1x10^6$ and $1x10^5$spores/ml according to **Admas *et al.* (2017)**.
Healthy water hyacinth plants with the broadest leaves having 50–100 cm^2 in size and of approximately the same age as determined by their architecture were collected from Kisumu City shoreline of Lake Victoria according to the method of **Kuzmenko (2016)** and **Mujere (2015)**. The sampled plants were put into the aged water to acclimatize for 2 days **(Piyaboon *et al*., 2016)** before being inoculated. The healthy plants were placed in 20 liter basins at the rate of 3 plants per basin. The plants were applied with the 6 treatments or formulations of *C. piaropi* and *M. roridum* with; $1x10^9$, $1x10^8$, $1x10^7$, $1x10^6$ and $1x10^5$spores/ml of each of the pathogens using 100mls of the formulation on the plants with a spray pump held at 20 cm from the plant and inclined at 45⁰ according to the method used by **Opande *et al*. (2013)**. The formulation with the lowest concentration ($1x10^5$spores/ml) was sprayed first and subsequent concentrations sprayed in ascending order. The leaves of the plants were fully wetted by the spray. The control plants were sprayed with sterile distilled water. To ensure sufficient moisture for infection, a fine mist of sterile water was sprayed upon the leaves after the formulation spray droplets had evaporated according to **Admas *et al*. (2017)**. The experimental setup was completely randomized design (CRD).

At weeks 2, 4, and 6 after inoculation, and following the method of **Sharma *et al*. (2016)**, the lengths of the three plants in each basin were individually measured. This was done using a centimeter ruler and the average for each basin recorded. The average shoot length for the treated basins was compared with the average length of the control basins. Relative shoot length for each treatment was determined by adopting the formula of **Robert and James (1991)** as follows:

$$R = \frac{yp - yt}{yp} X100$$

Where:
R = relative shoot length in water hyacinth
y*p* = average shoot length from the control treatment
y*t* = average shoot length from the respective treatments.

The relative shoot length for each treatment was therefore the percentage by which the average length of the inoculated shoots varied from the average shoot length of the control plants.

Following the method of **Daddy and Owotunse (2002)**, at the end of the sixth week the plants from each basin were removed from the water and the roots disentangled gently. The stalks were removed from the roots by hand and blotted with a serviette to remove excess water and immediately weighed on an electronic scale. Harvested leaves, stalks and whole plants were taken to the laboratory and oven dried at 80⁰C ffor 24 hours to a constant weight. The dry matter was removed from the oven and weighed. The plants from the control basin were also removed and subjected to the excess water removal, weighing, oven drying and weighing again. The weights of each treatment were subjected to comparison to the weight of the control treatment by calculating the relative biomass using the formula developed **by Robert and James (1991)** as follows:

$$I = \frac{Ap - At}{Ap} X100$$

Where:
I = relative biomass
Ap = water hyacinth dry weight from control treatment
At = water hyacinth dry weight from the respective treatment

The relative biomass for each treatment was therefore the percentage by which the average biomass of the inoculated shoots varied from the average biomass of the control plants.
Combined analyses were done with spore formulation treatments and pathogen effects considered on all the data using PRO GLM in SAS (Institute, Inc.1999).

3. Results and discussion

For both pathogens, as the concentration of spores increased, there was a corresponding significant ($p \le 0.05$) increase in relative shoot length (Table 1). *Cercospora piaropi* recorded significantly higher relative shoot length at all the spore concentrations. The highest relative shoot length for *C. piaropi* was 46.34 while for *M. roridum* was 41.80, both being recorded for $1x10^9$ spores /ml. In addition, the mean relative shoot length for *C. piaropi* was significantly higher at 41.31 as compared to that of *M. roridum* which was 38.51.

The increasing relative shoot length with increasing spore concentration for both pathogens suggested that the inoculated plants had suppressed shoot elongation as compared to the control plants. The significantly higher relative shoot length for *C. piaropi* as compared to *M. roridum* was compelling evidence to suggest that *C. piaropi* elicited a higher suppression of shoot length on water hyacinth than *M. roridum* did. The importance of these results was that both pathogens reduced growth and resurgence of the weed disallowing the potential of the weed to build huge populations that form dense mats on water surfaces. This was in conformity with the findings of **Asmare (2017)** and **Work and Ashlie (2018)** who reported similar results in Lake Tana. The results also agreed with the findings of **Doehlemann *et al.* (2017)** that fungal pathogens manipulate plant metabolism in their own favour therefore denying the plant the necessary resources for tissue growth with subsequent reduction on growth. The bio pathogens were thus seen as important in lessening the detrimental effects of the normally luxuriant water hyacinth growth in agreement with similar results with **Sharma *et al.* (2016)** and **Waithaka (2013)** who reported that reduction in shoot length is attributable to the severe stress caused by the pathogens, which affect the ability of the mature plants to produce strong fresh leaves and daughter plants.

It was observed that as the concentration level of the spores for both *C. piaropi* and *M. roridum* increased, there was a significant ($p \le 0.05$) increase in relative biomass. The highest relative biomass for the two pathogens were 73.53 for *C. piaropi* and 37.60 for *M. roridum* at $1x10^9$ spores/ml (Table 2). Comparison of the two pathogens with regards to relative biomass showed that *C. piaropi* had a significantly higher mean relative biomass at 64.81 as compared to 32.34 of *M. roridum.*

The increased relative biomass with increased spore concentration for both pathogens suggested that the inoculated plants had suppressed biomass accumulation as compared to the control plants. The significantly higher relative biomass for *C. piaropi* as compared to *M. roridum* was compelling evidence to suggest that *C. piaropi* elicited a higher suppression of biomass in water hyacinth than *M. roridum* did. The results were in agreement with the findings of **Admas *et al.* (2017)** who reported that fungal pathogens cause diseases upon water plants that reduce their biomass. These results also conformed to the findings of **Joost van den Brink *et al.* (2013)** who in a study of plant biomass degradation by *Myceliophthora heterothallica* reported that fungal pathogens are able to degrade the biomass of plants. The results further agreed with the findings of **Moran (2005)** who demonstrated similar results in field plots with *C. piaropi*. This lessened biomass curtailed interference of the weed and put it at manageable levels in accordance with **Eid and Shaltout (2017).** In addition, the results agreed with the findings of **Robles *et al.* (2015)** that biomass reduction is useful and effective as a control method for water hyacinth.

Table 1. Effect of corn oil formulations on relative shoot length of water hyacinth plants during the study period

Pathogen	Spore conc. (ml^{-1}) $1x10^5$	$1x10^6$	$1x10^7$	$1x10^8$	$1x10^9$	Mean
C. piaropi	38.89f	38.49c	40.95h	42.40k	46.34m	**41.31i**
M. roridum	35.69a	37.10b	38.70e	39.27g	41.80j	**38.51d**
%CV						16.9
LSD						0.78

Numbers followed by different letters are significantly different at $p \le 0.05$

Table 2. Comparative effect of the pathogens on relative biomass

Pathogen	Spore conc. (ml-1) $1x10^5$	$1x10^6$	$1x10^7$	$1x10^8$	$1x10^9$	Mean
C. piaropi	57.40d	60.77e	63.83f	68.53g	73.53h	**64.81f**
M. roridum	39.53c	24.73a	26.73a	33.13b	37.60c	**32.34b**
LSD						3.40
%CV						11.10

Numbers followed by different letters are significantly different at $p \le 0.05$

5. Conclusion

Of the two fungal pathogens *C. piaropi* and *M. roridum*, the former is the better bio control option. Its application at rates of $1x10^9$ spores/ml has the potential to lower water hyacinth shoot growth and biomass accumulation and can therefore be recommended to be used in water hyacinth management efforts.

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Declaration of interest

We, the authors report no conflict of interest and they are the only ones responsible for the content and writing of the paper.

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Antibacterial Activity of Selected Ethnomedicinal Plants Popular in Magar Ethnic Community of Palpa District, Western Nepal

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Abstract
The main objective of this research was to explore the potential antibacterial activity of 25 selected medicinal plant extracts against four strains of bacteria. The ethnomedicinal knowledge was documented using semi-structured, open-ended questionnaires, informal interviews, and group discussions with traditional healers and knowledgeable persons about plants and plant-based remedies. The evaluation of antibacterial activities of twenty-five extracts of different plants was carried out by adopting the disc diffusion method for four bacterial strains, namely - *Pseudomonas aeruginosa, Escherichia coli*, *Bacillus subtilis* and *Staphylococcus aureus*. The results were reported by observing the inhibition zones. The results indicated that among 25 plant extracts examined, 16 (64%) plant extracts showed antibacterial property against *Escherichia coli* followed by 15 (60%) plant extracts against *Pseudomonas aeruginosa*, and 19 (76%) extracts each against *Staphylococcus aureus* and *Bacillus subtilis*. Four plant extracts were not able to produce the zone of inhibition with any of the tested bacteria. Gram-positive bacteria are found to show more positive effects as compared to Gram-negative. Present findings of this study indicate that ethnomedicinal plant extracts have antibacterial activity against the different strains of tested bacteria. This activity supports their use in the treatment of infections caused by such resistant bacteria.

1. Introduction

The history of human civilizations and the development of economic systems and thoughts are all inherently and intricately interwoven with the biological resources (**Ravi & Pusphpagadan, 1997**). Plant resources are naturally precious for the synthesis of medicinal compound and provide great help in discovery in the area of the pharmaceutical field because of the unknown availability either as a standardized extract or as a pure compound (**Hassan &Ullaha, 2019**). Approximately 85,000 plant species are known to be medicinally useful in all over the world (**Liu & Wang, 2008**). Medicinal plants have been used for many centuries not only in rural areas but also increasingly by urban citizens in both developing and developed countries. Plants based primary healthcare customs have a long history for their uses in various human ailments. Being comparatively harmless, the naturally occurring plant species and their products have attracted the huge attention of modern researchers in the treatment of various challenging diseases (**Guna, 2018**). Use of herbal medicines in Nepal accumulates a long history of human interactions with the surrounding environment. Plants and their products-based, traditional medicine system continues to contribute to the role of an important part in health care, with about 80% of the world's inhabitants relying mainly on traditional medicines for their primary health care (**Raja et al., 2011; Abraham and Thomas, 2012**).
Over the last decades, significant amount of evidences have emerged indicating that chemically diverse classes of plant secondary metabolites are of potential interest for therapeutic interventions in several human diseases (**Napagoda et al., 2020**). Considering the high costs of the synthetic drugs and their various side effects, the search for alternative products from plants used in folklore medicine is further investigated (**Kamaraj et al., 2012**). Initially, the development of novel drugs was primarily through the extraction of biologically active compounds from plants which were identified through medicinal use or a variety of bioactivity screening tests (**Hunter, 2001**). Most of the drugs currently used to treat bacterial and other infections were first isolated from natural sources including ethnomedicinal plants (**Coe &Anderson, 1996; Bhattarai & Basukala, 2016**). The medicinal values of plants lie in some chemical substances that produce a definite physiological action on the human body. The most important of these bioactive compounds of plants are alkaloids, flavonoids, tannins, and phenolic compounds. Various herbal species have

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been known to display antimicrobial properties by acting against foodborne pathogens and spoilage bacteria and be used as sources of natural antimicrobial substances for the treatment of infectious diseases (**Nabaviet al., 2015**). The documentation of several ethnomedicinal uses of plants and indigenous knowledge has been carried out at different corners of Nepal.

However, in Nepal, the investigation of ethnomedicinal plants used by various indigenous and local communities to correlate with antibacterial activities, the works are still on the way of exploring and only a few research work have been demonstrated by the researchers(**Taylor & Towers, 1998; Parajuliet al., 2001; Sharma et al., 2002; Taylor et al., 2002; Vaidya et al., 2006; Bhattaraiet al., 2008; Shakya et al., 2008; Bhattarai & Basukala, 2016**).The current investigation aims to evaluate the *in-vitro* antibacterial activity of some selected ethnomedicinal plants explored from different regions of Purbakhola Rural Municipality of Palpa District for the first time to assess their potential antibacterial properties.

To answer the research questions for our work, whether all the medicinal plants used by indigenous (including Magar ethnic community) and local people show bioactivity against pathogenic bacteria or not? We focus to access the *in-vitro* antibacterial activity of selected ethnomedicinal plant species in the Palpa District in province number five of Nepal. In the present research, a total of 25 selected ethnomedicinal plants were examined for their antibacterial properties *in-vitro* using the disc diffusion method.

2. Material and methods

2.1 Collection and Processing of ethnomedicinal plant species

Ethnomedicinal plant species with the use of their particular parts were collected and their indigenous knowledge has been documented from different villages of Siluwa-1 and Ringneraha-3 of the Purbakhola Rural Municipalityof Palpa District in Province Number five of Nepal (Figure 1). The villages surveyed were Arkhaldanda, Koranga, and Nandedanda of Ringneraha ward no. 3 and five villages in Siluwa ward no. 1 were Dhakrebash, Arghichaur, Gundanda, Hattilek, and Tarepahad.

The collected voucher herbarium specimens were identified and authenticated with the help of Standard literatures (**Balley, 1969; Hooker, 1872-1897; Polunin & Stainton, 1987, Stainton, 1988; Grierson & Long, 1983-2001**). A set of voucher herbarium specimens was made for each collection and their numbers are listed in Table1, and were deposited at the Tribhuvan University, Central Herbarium (TUCH), Nepal. Selected samples were based on the use of local people that were repeatedly used to treat the same illness by several traditional healers, villagers, and traders. The plants were dried at room temperature for two weeks.

Information about the ethnomedicinal utilization of plants and their products was gathered by interviewing knowledgeable persons and local faith healers according to previous works (**Bhattarai et al., 2009; Pangeni, 2009; Bhattarai et.al., 2010**).

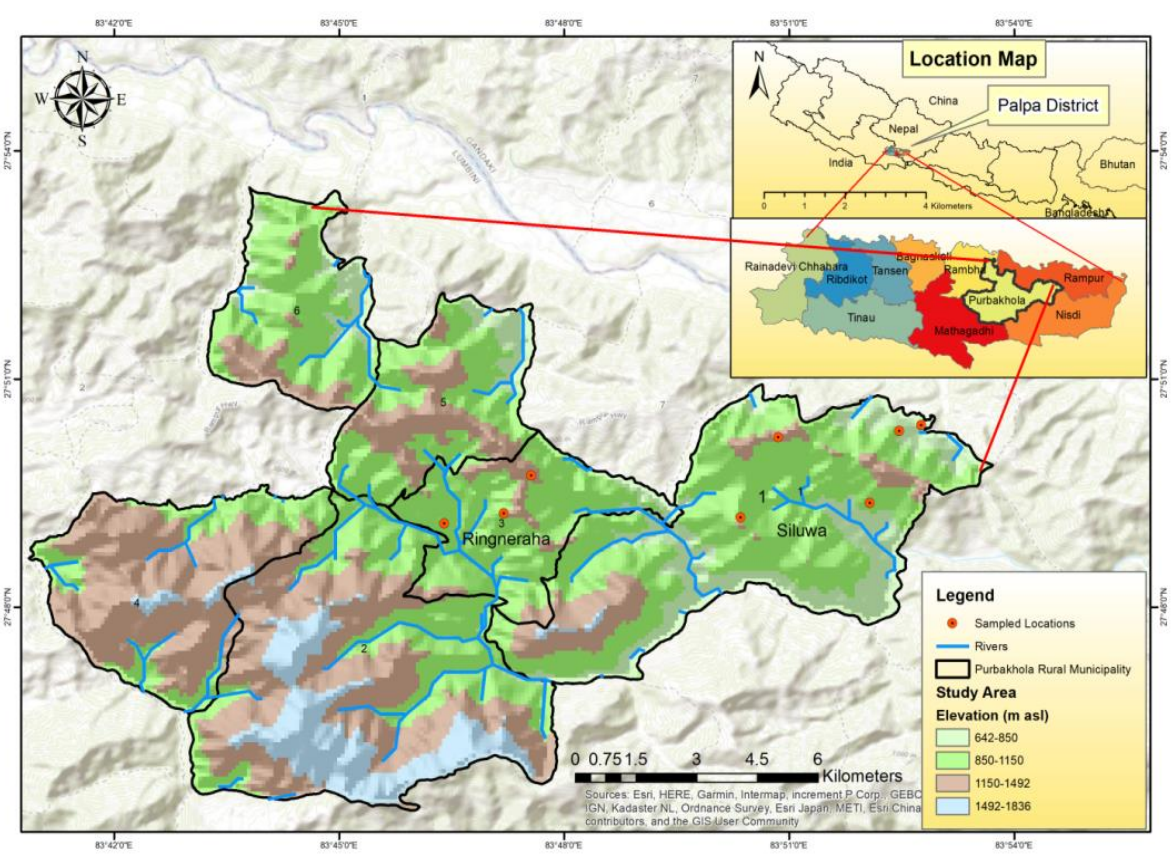

Figure 1. Map of the study area showing Purbakhola Rural Municipality (Source: **Pangeni et al., 2020**)

2.2 Preparation of plant extracts

Plant samples for laboratory investigation were air-dried in the shade at room temperature and stored in cotton bags for diffusion tests. They were stored in a dark and cool place to minimize chemical degradation. The plant extracts were prepared following published papers (**Taylor & Towers 1998; Parajuli et al., 2001; Taylor et al., 2002**) with some minor modifications. The plant parts were ground and then 2 g sample powder of each plant material was immersed in 25 mL methanol (MeOH) for 24 hours. The sample was then extracted using suction-filtered through Whatman number 1 filter paper and the

residue was again immersed with another 25 mL MeOH for the next 24 hours. This process was continued until the extract was turning into colorless. The filtrates were then dried with the help of an electric table fan. After being complete dryness of samples, the extract was re-suspended in 2 mL of methanol. The final concentration of the extract was 1g dried plant material per mL methanol. A paper disk of 6 mm diameter was prepared from whatman filter paper no.1. Three types of test disks were made by using tetracycline (positive control-test disk dipped in 0.25 $mgmL^{-1}$ tetracycline), methanol (negative control-paper disk dipped in MeOH), and plant extracts (test disk dipped into plant extract). Thus, formed all the disks were allowed to dry at room temperature for antibacterial testing.

2.3 Bacterial Strains used

A total of four bacterial species including two Gram-negative (*Pseudomonas aeruginosa* and *Escherichia coli*) and two of Gram-positive (*Bacillus subtilis* and *Staphylococcus aureus*) were used for this study. These bacterial strains were kindly received from the Central Department of Microbiology, Tribhuvan University, Kirtipur, Kathmandu, Nepal. Inoculums of each bacterial strain was suspended in 5 mL of nutrient broth and incubated overnight at 37°C. These cultures were diluted (1/10) with nutrient broth before use.

2.4 Antibacterial Activity

The disc diffusion method was adopted to screen the antibacterial activity (**Taylor, Manandhar & Towers, 1995; Bhattarai et al., 2008**). The *in-vitro* antibacterial property was carried out by using standard sterile filter paper disks of 6 mm saturated with plant extracts. Overnight cultures were prepared by suspending 3-4 isolated colonies in 5mL of nutrient broth and incubating for 24 hours at 37⁰C. The overnight culture was used to inoculate the nutrient agar test plates. The test plates were inoculated with a suitable bacterial overnight culture medium on a sterile cotton swab. After inoculation, the test disks and the control disks were added. These plates were incubated upside down for about 24 hours at room temperature. Finally, the results were recorded as the presence or absence of a zone of inhibition, and testing was repeated for at least three times to ensure the reliability of the laboratory results.

3. Results

The information about the uses of ethnomedicinal plants by indigenous people (including Magars) and local communities from the study area by interviewing the faith healers, knowledgeable persons, and elder people are compiled in Table 1. The main outcomes of laboratory testing are summarized in Table 2. Twenty-five species of ethnomedicinally used plant extracts were examined; out of them, sixteen plant species showed antibacterial property i. e. produce a clear zone of inhibition against *Escherichia coli* (64%). The species were *Acorus calamus, Aesandra butyracea, Amaranthus spinosus, Anemone vitifolia, Bergenia ciliata, Cassia fisula, Centella asiatica, Cissampelos pareira, Clerodendrum viscosum, Curcuma amada, Eclipta prostrata, Fragaria nubicola, Oxalis corniculata, Rhododendron arboreum, Swertianervosa* and *Woodfordia fruticosa*, Nineteen extracts (*Acorus calamus, Amaranthus spinosus, Anemone vitifolia, Asparagus racemosus, Bergenia ciliata, Cassia fistula, Centella asiatica, Clematis buchananiana, Clerodendrum viscosum, Curcuma amada, Eclipta prostrata, Fragaria nubicola, Mallotus philippensis, Oxalis corniculata, Rhododendron arboreum, Solanum torvum, Swertia nervosa, Woodfordia fruticosa* and *Zingiber officinale*) showed positive effects against *Staphylococcus aureus* (76%).
Similarly, fifteen plant extracts (*Acorus calamus, Anemone vitifolia, Asparagus racemosus, Bergenia ciliata, Cassia fistula, Centella asiatica, Clerodendrum viscosum, Curcuma amada, Fragaria nubicola, Mallotus philippensis, Oxalis corniculata, Rhododendron arboreum, Swertia nervosa, Woodfordia fruticosa* and *Zingiber officinale*) exhibited antibacterial property against *Pseudomonas aeruginosa* (60%), and 19 plant extracts (*Acorus calamus, Centella asiatica, Rhododendron arboreum, Bergenia ciliata, Oxalis corniculata, Fragaria nubicola, Swertia nervosa, Curcuma amada, Cassia fistula, Anemone vitifolia, Clerodendrum viscosum, Woodfordia fruticosa, Aesandra butyracea, Cissampelos pareira, Clematis buchananiana, Mallotus philippensis, Zingiber officinale, Asparagus racemosus, Amaranthus spinosus*) showed positive results against *Bacillus subtilis* (76%) (Figure 2).
The examined plant extracts produced clear zone of inhibition recording a higher percentage in Gram-positive bacteria (*Staphylococcus aureus* and *Bacillus subtilis*,76%) than in Gram-negative bacteria (*Escherichia coli,* 64% followed by *Pseudomonas aeruginosa*, 60%) (Figure 3). The results were found similar following the previous research work (**Mccutcheon et al., 1992; Bhattarai et al., 2009; Bhattarai & Basukala 2016; Gupta et al., 2016**). These outcomes may be due to the presence of a more complex cell wall in the structure of Gram-negative bacteria.

4. Discussion

Twenty-five extracts of different types of medicinal species were screened for their antibacterial property against four strains of pathogenic bacteria which can cause various diseases like cuts and wound, fever, diarrhea, dysentery, sinusitis, tonsillitis, pneumonia, urinary problems, respiratory ailments, boils, etc. The extracts were made from different parts of the medicinal plant like root, fruit, leaves, stem, bark, flower, inflorescence, seed, etc. The plant species that were used to treat various ailments/diseases in traditional herbal remedies potentially caused by human pathogenic bacteria, were examined.
Out of 25 medicinal plant extracts, tested, four plant species namely: *Artemisia dubia*, *Corchorus aestuans*, *Eryngium foetidum*, and *Lobelia pyramidalis* were unable to show the zone of inhibition towards any one of the tested bacteria. The results do not mean that the medicinal plants were valueless for traditional practice. There may be several possible reasons for this, as the polarity nature of methanol solvent made most of the plant species to show zone of inhibition in tested pathogenic bacteria and the methanol solvent was selected as it is easily available in the market. In the present investigation, we used an only little number of bacteria for the bioassay process; it may be also considered that the medicinal plants used here may contain antibacterial properties against pathogenic bacteria other than those tested, or the solvent used was unable to extract the active constitutes.
Gram-positive bacteria were found as more active to show more comparable results in tested extracts than that of Gram-negative bacteria. In the current research, Gram-positive bacteria (*Staphylococcus aureus* and *Bacillus subtilis*) were found to show the more positive and sensitive effect towards 38 plant extracts among 50 samples (76%), tested. Similarly, Gram-negative bacteria (*Escherichia coli* and *Pseudomonas aeruginosa*) were found to show the positive effect for 31 plant extracts (62%) (Figure 3). As clear from the figure, the difference in showing positive effects for several extracts, between Gram-positive and Gram-negative bacteria can be well described to the morphological differences between these microorganisms and mostly to the differences in the permeability of the cell wall (**Bereksi et al., 2018**).

Table 1. Traditional use of ethnomedicinal plants in Palpa district

Plant scientific name	Family	Parts used	Origin/Voucher specimen	Traditional uses
*Amaranthus spinosus*L.	Amaranthaceae	Leaves, stem	Wild/cultivated [B.Pangeni 24, TUCH]	Boils, burns, cough, cold, dizziness
*Acorus calamus*L.	Araceae	Root	Cultivated [B.Pangeni 323,TUCH]	Cough,sore throat
*Aesandra butyracea*Roxb.	Sapotaceae	Bark, seeds	Wild [B.Pangeni 303, TUCH]	Sinusitis, stomachache
Anemone vitifolia Buch.-Ham. ex DC.	Ranunculaceae	Root, leaves	Wild/[B.Pangeni 12, TUCH]	Dysentery, dandruffs
*Artemisia dubia*Wall ex Besser	Asteraceae	Leaves, root	Wild/[B.Pangeni 317, TUCH]	Cutsand wound, asthma
Asparagus racemosus Willd.	Liliaceae	Tuber, shoot	Wild/Cultivated [B.Pangeni 360, TUCH]	Fever, urinary troubles
Bergenia ciliata(Haw.)Sternb.	Saxifragaceae	Rhizome	Wild [B.Pangeni 355, TUCH]	Rheumatism, diarrhea and dysentery
Cassia fistula L.	Fabaceae	Fruit, seeds, root	Wild [B.Pangeni 306, TUCH]	Fever, tonic and diabetes
Centella asiatica(L.) Urb.	Apiaceae	Whole plant	Wild [B.Pangeni 06, TUCH]	Stomachache, indigestion, asthma and gastric
Cissampelos pareira L.	Menispermaceae	Rhizome, Leaves	Wild [B.Pangeni 328, TUCH]	Malarial fever, common cold and cough
Clematis buchananiana DC.	Ranunculaceae	Leaves	Wild [B.Pangen 14a, TUCH]	Sinusitis
*Clerodendrum viscosum*Vent.	Verbenaceae	Seed, leaves	Wild/Cultivated [B.Pangeni 348, TUCH]	Gastric, stomachache
*Corchorus aestuans*L.	Tiliaceae	Whole plant	Wild/Cultivated [B.Pangeni 05,TUCH]	Fever, production of milk for women after post-delivery
*Curcumaamada*Roxb.	Zingiberaceae	Rhizome	Cultivated [only observed]	Skin allergy
Ecliptaprostrata(L.)L.	Asteraceae	Aerial parts	Wild/Cultivated [B.Pangeni 09,TUCH]	Diarrhea and Dysentery
Eryngium foetidum L	Apiaceae	Leaves	Cultivated [B.Pangeni 320, TUCH]	Headache
Fragaria nubicola Lindl.ex Lacaita	Rosaceae	Whole plant	Wild/Cultivated [B.Pangeni 308,TUCH]	Dysentery
*Lobelia pyramidalis*Wall	Lobeliaceae	Leaves, inflorescence	Wild [B.Pangeni 338, TUCH]	Asthma, bronchitis
Mallotus philippensis (Lam.) Mull-Arg.	Euphorbiaceae	Bark	Wild [B.Pangeni 301, TUCH	Diarrhea and dysentery
Oxalis corniculata L.	Oxalidaceae	Whole plant	Wild [B.Pangeni 313, TUCH]	Eye infection
Rhododendron arboreum Sm.	Ericaceae	Flower	Wild/Cultivated [B.Pangeni 345, TUCH]	Dysentery
*Solanum torvum*Swartz.	Solanaceae	Fruit	Wild [B.Pangeni 305, TUCH]	Headache, dizziness
Swertia nervosa (G.Don) C.B. Clarke	Gentianaceae	Whole plant	Wild/cultivated [B.Pangeni 14b,TUCH]	Diarrhea, stomachache, tonic
Woodfordia fruticosa(L.) Kurz	Lythraceae	Flower	Wild [B.Pangeni 300,TUCH]	Dysentery, stomachache
Zingiber officinale L.	Zingiberaceae	Rhizome	Cultivated [only oberved]	Diarrhea, common cold and cough

Table 2. Antibacterial activities of Medicinal Plants with different strains of bacteria

Plant scientific name	Results of bioassay test			
	Escherichia coli	***Staphylococcus aureus***	***Pseudomonas aeruginosa***	***Bacillus subtilis***
Tetracycline (Positive control)	+	+	+	+
Methanol (Negative control)	-	-	-	-
*Amaranthus spinosus*L.	+	+	-	+
Acoru scalamus L.	+	+	+	+
Aesandra butyracea Roxb.	+	-	-	+
*Anemone vitifolia*Buch.-Ham. ex DC.	+	+	+	+
*Artemisia dubia*Wall ex Besser	-	-	-	-
*Asparagus racemosus*Willd.	-	+	+	+
Bergenia ciliata (Haw.) Sternb.	+	+	+	+
Cassia fistula L.	+	+	+	+
Centella asiatica (L.) Urb.	+	+	+	+
*Cissampelos pareira*L.	+	-	-	+
Clematis buchananiana DC.	-	+	-	+
*Clerodendrumviscosum*Vent.	+	+	+	+
Corchorus aestuans L.	-	-	-	-
*Curcuma amada*Roxb.	+	+	+	+
Ecliptaprostrata(L.) L.	+	+	-	-
Eryngium foetidum L	-	-	-	-
Fragaria nubicola Lindl. ex Lacaita	+	+	+	+
*Lobelia pyramidalis*Wall	-	-	-	-
Mallotus philippensis (Lam.) Mull-Arg.	-	+	+	+
Oxalis corniculata L.	+	+	+	+
Rhododendron arboreum Sm.	+	+	+	+
Solanum torvum Swartz.	-	+	-	-
Swertia nervosa (G.Don)C.B. Clarke	+	+	+	+
Woodfordia fruticosa(L.) Kurz	+	+	+	+
Zingiber officinale L.	-	+	+	+

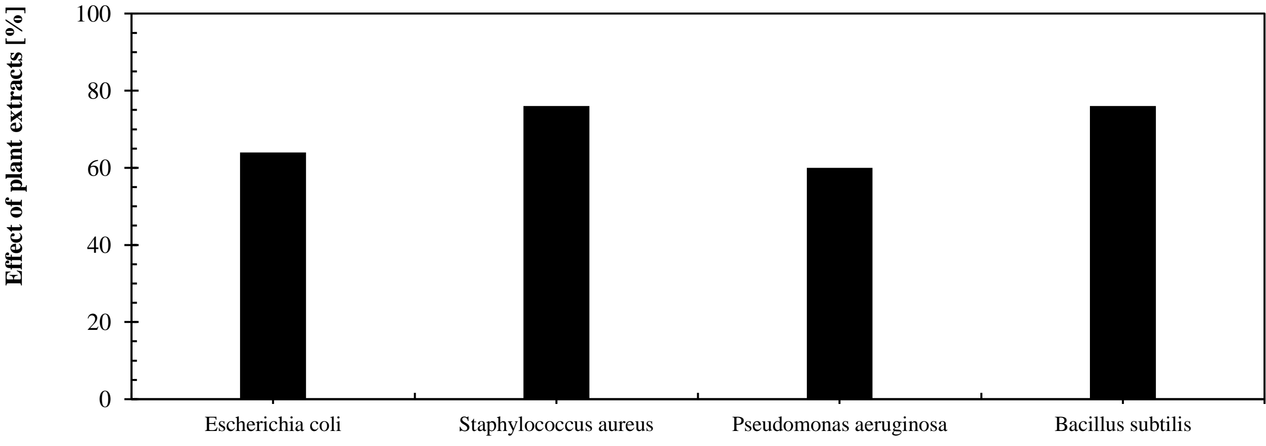

Figure 2. Antibacterial activity of medicinal plant extracts from Palpa District against the tested bacterial strains

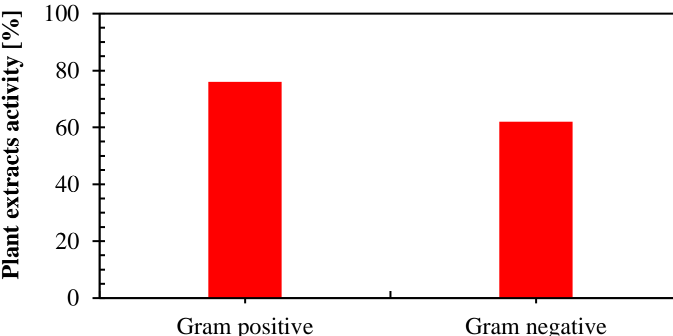

Figure 3. Percentage of plant extracts showing inhibitory activity against tested bacterial strains.

Among the medicinal plants tested, 12 plant extracts (*Centella asiatica*, *Rhododendron arboreum*, *Bergenia ciliata*, *Acorus calamus*, *Oxalis corniculata*, *Fragaria nubicola*, *Swertia nervosa*, *Curcuma amada*, *Cassia fistula*, *Anemone vitifolia*, *Clerodendrum viscosum*, *Woodfordia fruticosa*) showed the most promising antibacterial properties with all the four tested strains of bacteria (*Staphylococcus aureus*, *Bacillus subtilis, Escherichia coli*, and *Pseudomonas aeruginosa*), indicating the potential for discovery of antibacterial principles. Four plant extracts (*Aesandra butyracea*, *Cissampelos pareira*, *Clematis buchananiana*, and *Eclipta prostrata*) showed positive results for only any two of the tested bacteria (i. e. *Aesandra butyracea* and *Cissampelos pareira* with *Bacillus subtilis* and *Escherichia coli*; *Clematis buchananiana* with *Bacillus subtilis* and *Staphylococcus aureus*; *Eclipta prostrata* with *Escherichia coli* and *Staphylococcus aureus*). Similarly, plant extract (*Solanum torvum*) showed positive effect with only one of the tested bacteria (*Staphylococcus aureus*), four plant extracts (*Amaranthus spinosus*, *Asparagus racemosus*, *Mallotus philippensis* and *Zingiber officinale*) with any three of the tested bacteria (i.e. *Mallotus philippensis*, *Asparagus racemosus* and *Zingiber officinale* with *Staphylococcus aureus*, *Pseudomonas aeruginosa* and *Bacillus subtilis*; *Amaranthus spinosus* with *Escherichia coli*, *Staphylococcus aureus* and *Bacillus subtilis*), and four plant species extracts (*Artemisia dubia*, *Corchorus aestuans*, *Eryngium foetidum* and *Lobelia pyramidalis*) with none of the tested bacteria. The results also indicate that scientific research conducted on medicinal plants having traditional claims of effectiveness might correlate with laboratory tests results.
Extracted plant medicines are safe, effective, cheaper, and have no or little side effects (**Hassan & Ullah, 2019**). The active compounds (phytochemicals) are responsible for biological activity such as antibacterial against infectious pathogens and provide a quite significant role in the discovery of new antibiotic herbal medicines. The present study investigated the antibacterial potential of a medicinal plant for the first time in the Purbakhola Rural Municipality of Palpa District in Province number five of Nepal.

5. Conclusion

Thus, it may be concluded that due to the presence of useful phytoconstituents in the tested plant extracts towards their antibacterial properties for four strains of bacteria, they show quite significant and clear zone of inhibition, therefore, these traditional medicinal plants could be used as potent sources of natural antibacterial agents as a substitute for the commercially available synthetic drugs which are quite expensive and may have a large number of side effects. Further phytochemical studies are required to determine the type of compounds responsible for the antibacterial effects of these species. Further extensive research is also required for the separation and recognition of active biomolecules and principles present in these extracts so that they could be utilized for the pharmaceutical purpose at the industrial scale.

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Declaration of interest

The authors report no conflict of interest.

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Evaluation of Moringa Peregrina (Forsk) Fiori, Leaf and Seed Extract Against Multidrug Resistant Strains of Bacteria and Fungus of Clinical Origin

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Abstract

The emergence of antibiotic resistant microorganism strains has become a critical concern in the treatment of infectious diseases and makes the search of an alternative therapy a must. The study was designed to evaluate the in vitro antimicrobial activities of the *Moringa peregrina* (MP) leave (MPL) and seed (MPS) extracts. Antimicrobial assays were performed using a microplate growth inhibition assay against 11 multidrug-resistant (MDR) strains. Following qualitative analysis, dose-response assays were performed using the MTT colorimetric assay. The results showed a strong correlation between the MPL and MPS extract concentration and growth inhibition (P<0.001). MP extract revealed a remarkable antimicrobial effect and inhibited the growth and survival of MDR pathogens which include *Escherichia coli; Pseudomonas aeruginosa; Klebsiella pneumonia; Acinetobacter baumannii; Staphylococcus aureus* between (88.6-94.7 %) and between (62.3- 88.7%) against *Candida Kefyer; Candida parapsilosis; Candida albicans; Candida glabrata; Aspergillus flavus and Fusarium oxysporum*. MIC_{50} ranging from ≤6.25 to 25 mg/mL. *Acinetobacter baumannii* and *Pseudomonas aeruginosa* were the most susceptible to MP extracts (MIC50 < 6.25 mg/mL). These results support the use of MP in Arab traditional medicine as natural antimicrobial agents. Additionally, the use of such naturally occurring adjuvant derived from medicinal plants can be used as an adjuvant with synthetic antibiotics to combat bacterial resistance and to enhance the antibacterial potential. Further studies are recommended on isolation and purification of novel antimicrobial molecules to treat the infections caused by microbes.

1. Introduction

Worldwide, infectious diseases are a significant cause of morbidity and mortality with the World Health Organization (WHO) estimating it to account for 50% of all deaths in tropical countries. The current increase in health casualties associated with bacterial or fungal infections is because of treatment failures related to the growing bacterial resistance to most anti-infective agents that greatly lessen their efficacy **(Tchana *et al.*, 2014)**. Thus, widespread multidrug-resistant (MDR) strains of bacteria necessitate a regular substitute of new drug sources for the effective treatment of infectious diseases **(Khan *et al.*, 2009)** which includes newer classes of antibacterial from either synthetic or natural sources and inhibit these resistance mechanisms **(Soares *et al.* 2019)**. One of the most effective, safe and reliable sources of antimicrobial agents are medicinal plants and their metabolites or derivatives **(Lalas *et al.*, 2012)**. For instance, an appropriate combination of antibiotics and natural antimicrobial substances are potential approaches for combating MDR microorganisms **(Breijyeh *et al.*, 2020)**. Hence, there is a great need for new sources of antimicrobial agents to fight MDR strain infection and the WHO has recommended the member states to develop effective drugs to fight against this issue **(Silver and Bostian, 1993)**. The genus Moringa, called miracle tree is a member of the family Moringaceae. The species *Moringa peregrina* (MP) drumstick or alyusr tree is widely grown and cultivated in Saudi Arabia. MP has been used since ancient times and in many culture and traditions as a food as well as a medicinal plant owing to its medicinal value. All parts of *M. peregrina* are known to possess antibacterial activity **(Saleh *et al.*, 2017)**. The leaves and roots decoction is used for the treatment of malarial fever, stomach ailments, to regulate and control high blood pressures and hyperglycemia **(Elbatran *et al.*, 2005)**. The tender leaves are used to accelerate wound healing process **(Mekonnen *et al.*, 1999)**. In Saudi Arabian folk medicine, *M. peregrina* is used for the treatment of various disease conditions such as skin diseases, respiratory troubles, oral and ear infections, diabetes, anaemia and certain **cancers (Nawash & Al-Horani, 2011; Patel *et al.*, 2010; Emmanuel *et al.*, 2014; Kalkunte *et al.*, 2006; Jung *et al.*, 2014; Tiloke *et al.*,2013)**. In some earlier studies, various parts of the MP have been shown to possess antibacterial activity **(Al_husnan and Alkahtani, 2016)**. In recent years, *M. peregrina* is gaining added recognition due to its traditional, nutritional, industrial and medicinal values. Since various parts of this plant have a wide range of therapeutic uses, thus, it has been biologically screened

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(Anwar and Rashid, 2007). Earlier, an anti-cancer potential of Moringa leaves and bark extract has been reported **(Al Asmari *et al*., 2015)**. Moreover, a broad range of biological activities are also attributed to Moringa Spp. **(Senthilkumar *et al*., 2018)**; such as antidiabetic **(Rao *et al*., 2001)**, antispasmodic **(Sadraei *et al*., 2015)**, antioxidant **(Taniyama & Griendling, 2003), antimicrobial (Saleh *et al*., 2017)**, anti-hepatotoxic **(Elabd *et al*., 2017)**, anticholesterolemic **(Rouhi-Broujeni *et al*., 2013)**, anti-gastric ulcer (**Senthilkumar *et al*., 2018)**, anti-phlogistic **(Koheil *et al*., 2011)**, and memory-enhancing activities **(Elsaey *et al*., 2016)**. Although, various biological activities of *M. peregrina* have been exhaustively studied; the present investigation was undertaken of locally grown *Moringa peregrina* ethanol extract of leaves and seed kernel for their antimicrobial activity on multidrug-resistant bacteria and fungi isolates.

2. Material and methods

2.1 Plant material collection and extraction

The fresh leaves (L) and seeds (S) of *Moringa peregrina* were collected from Riyadh area. They were identified and authenticated by an expert taxonomist and a voucher specimen has been deposited at the CAM division of the center for future reference.

2.1.1 Preparation of leaves extract
Shade dried leaves of MP were coarsely powdered in an electric blender; extracted with 96% ethanol using soxholet apparatus. The solvent was evaporated under reduced pressure using a rotary evaporator (Buchi, Switzerland) to get semi solid viscous mass. Extract thus obtained was preserved at 4°C until further use.

2.1.2 Preparation seed extract
The dried MP seeds coat were removed manually and grinded to powder, soaked in 96% of ethanol and continuously shaken for 24 h. The filtrate was collected and filtered through Whatman filter paper. The solvent was eliminated under reduced pressure using rotary evaporator (Buchi, Switzerland).

2.2 Evaluation of antimicrobial activity

In this study, we focused upon the effect of *Moringa peregrina* extracts from leaves (MPL) and seeds (MPS) to observe its efficacy as an anti-microbial agent on a panel of 5 bacterial strains belonging to Gram-negative and positive bacteria including multidrug-resistant strains *Escherichia coli* (*E. coli*) ATCC 25922, *Pseudomonas aeruginosa* (*P. aeruginosa*) ATCC27853, extended spectrum beta lactamase producing *Klebsiella pneumoniae* (*K. pneumoniae*) ATCC 700603 and *Acinetobacter baumannii* (*A. baumanii*) clinical isolate and multidrug resistant *Staphylococcus aureus* (*S. aureus*) ATCC 25923; and (4) Pathogen yeast including *Candida Kefyer* (*C. kefyer*), *Candida parapsilosis* (*C. parapsilosis*) *Candida albicans* (*C. albicans*) and *Candida glabrata* (*C. glabrata*); and 2 molds including *Aspergillus flavus* (*A. flavus*) and *Fusarium oxysporum* (*F. oxysporum*). The ATCC strains were obtained from the American Type Culture Collection. *Acinetobacter baumannii*, and candida species were clinical isolates, a generous gift from the Medical Microbiology Division, Prince Sultan Military Medical City, Riyadh, Saudi Arabia.

2.2.1 Bacterial strains and antimicrobial activity
Bacterial species were Sub-cultured and maintained in Tryptic Soy Broth (TSB; Oxoid Ltd, Basingstoke, UK). at 4ºC while, yeast was sub-cultured and maintained in Sabouraud Broth (SB; Oxoid Ltd, Basingstoke, UK) at 4ºC. The microbial inoculums (bacterial, fungal) were prepared from an overnight culture, diluted in 0.85% NaCl to achieve 0.5 McFarl and (108 cells/mL). The suspension inoculum was carried out in MicroScan Inoculum water (Siemens Healthcare Diagnostics Inc. USA) from a colony alone. This suspension, after shaking in vortex by 15s was adjusted to 0.5 of McFarland scale, resulting in a concentration of $1x10^6$ CFU/mL, diluted, 1:10 in TSB for bacterial strains and in RPMI 1640 medium with GlutaMAX™ supplement (Gibco, Life Technologies, NY, USA) for yeast and fungi strains. The *Moringa pregrina* extracts dissolved in 2.5% dimethyl sulfoxide (DMSO), which is maximum volume of DMSO that could be used to dissolve solid extracts, were first dilution to the final concentration (200 mg/ml) for each extract and then serial two-fold dilution was made in concentration range 10~200 mg/ml in 10 ml sterile test tube containing 2.5% DMSO. The solvent DMSO (2.5%) that would not inhibit growth of the microorganisms was used as the negative control for all the experiments.

2.2.2 *Agar dilution method*
Two-fold serial dilutions of *Moringa peregrina* ethanol extract (leaves, barks and seeds) were made in molten TSA or SDA medium cooled down to 45°C to obtain the desired final concentrations. Bacterial suspensions (0.1 mLwith10^6 CFU/mL) were then inoculated on solid TSA or SDA. Agar plates were incubated aerobically at 37°C for 48 h for all tested organisms. Negative controls included ethanol in amounts corresponding to the highest quantity present in the agar dilution assay. Inoculated agar plates without added plant extract served as positive controls and the negative control contained DMSO in the concentration used to dilute the extracts. As antimicrobial control, we used three agents of different classes: Amphotericin B, Ampicillin and Gentamicin.

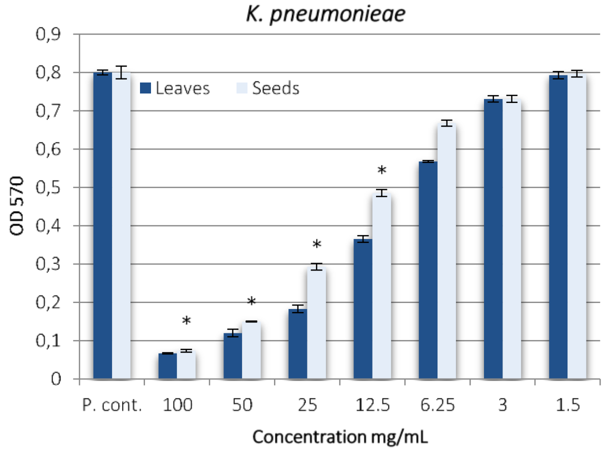
2.2.3 Growth inhibitory assay
In vitro susceptibility of bacterial and yeast isolates was performed using broth micro-dilutions according to the methodology recommended by the Clinical and Laboratory Standards Institute – CLSI in M27-A3 protocol (2008) and NCCLS/CLSI in M2–A9 and M7–A7 (2007). The cells were seeded in 96-well plates at a density of 3×10^8 cells/well and treated with *M. peregrina* leave and seed extracts concentrations ranging 1.5-100 mg/ml, and incubated at 35 ± 2°C, for 24 h. Microbial growth was detected former by optical density at the end of the exposure period and after by addition of 20 µL of a solution at 5 mg/mL of 3-(4, 5-imethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) to each single well and incubation for another 3 h at 35°C. The plates were read at absorbance of solubilized MTT in (HIDEX Oy, Turku, FINLAND) at 570 nm. Positive growth controls were inoculated as described above, without addition of plant extract. Viable bacteria are quantified by measuring cleavage of the yellow tetrazolium salt MTT into purple formazan in the presence of metabolically active bacterial/fungal cells. Inhibition (100%) was calculated as follows: [(Initial control absorbance - final absorbance) / (Initial control absorbance)] x 100. Determination of the concentration of MP extract causing 50% inhibition (MIC50) in reduction of the dye was calculated from the regression curve generated using GraphPad Prism 5.01 (GraphPad Software Inc., San Diego, CA). The results were recorded as means ± SE of the triplicate experiment.

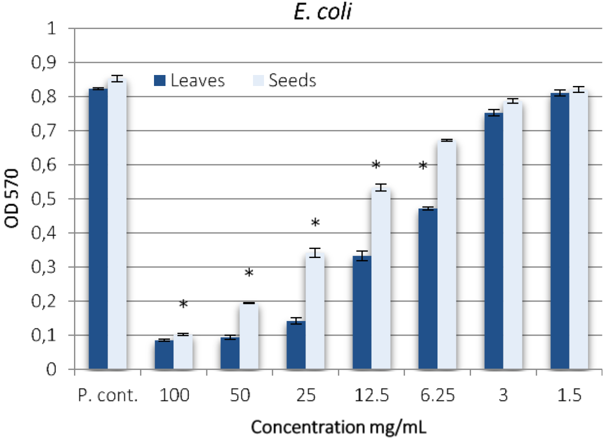
2.3 Data analysis

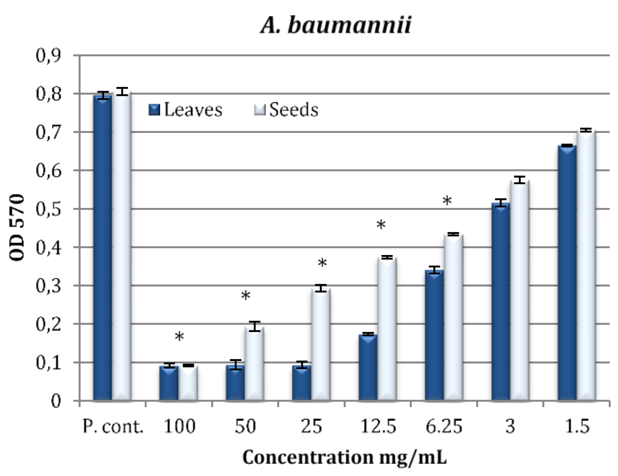
The results were expressed as mean ± standard error of mean (SEM), and statistical comparisons were made using analysis of variance (ANOVA) by Tukey test to compare means. A value of P≤0.05 indicated significance.

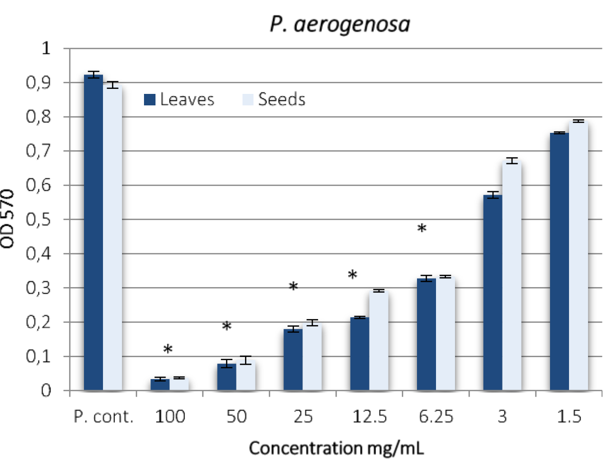
3. Results and Discussion

The results of this study validates the effects of ethanol extract of the leaves and seeds of *Moringa peregrina* (MP) as antimicrobial agents to fight various pathogens in Saudi folk medicine. In the present investigation, ethanol extracts of *Moringa peregrina* leaves and seeds clearly showed favorable antibacterial and antifungal activities on the tested bacteria including Gram-negative and Gram-positive and multidrug-resistant bacterial strains using various techniques. The antibacterial activity of leaf and seed extracts have exhibited significant inhibitory effects on the growth of a wide range of microorganisms. Surprisingly, the moringa extracts used in this study was found to be more effective than synthetic antibiotics. The obtained data on the growth of 11 multidrug-resistant (MDR) pathogens and yeast showed significantly potent antibacterial activity, this inhibitory effect recorded as dose-dependent in the culture media. Results are shown in the figure 1 & figure 2.









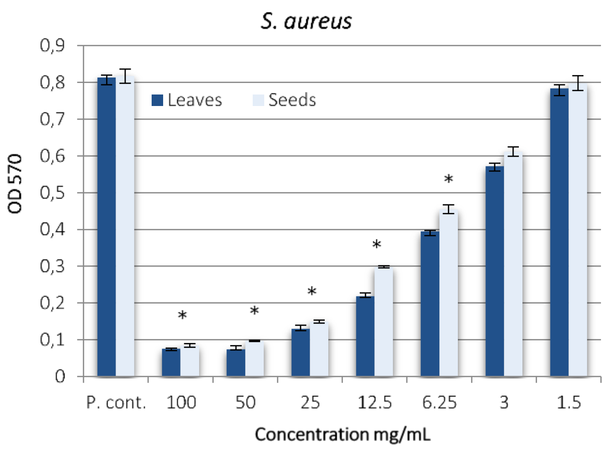


Figure 1. Antibacterial activity of *Moringa peregrina* leaves and seeds extracts against multi-drug resistant bacteria *K. pneumonieae; A. baumannii; S. aureus; E. coli* and *P. aeruginosa.* The values are optical density (OD) read at 570 nm expressed as mean ± standard error of the mean (S.E.M.) of three experiments. Star (*) denote statistically significant differences $p \leq 0.001$, compared with control group (untreated).

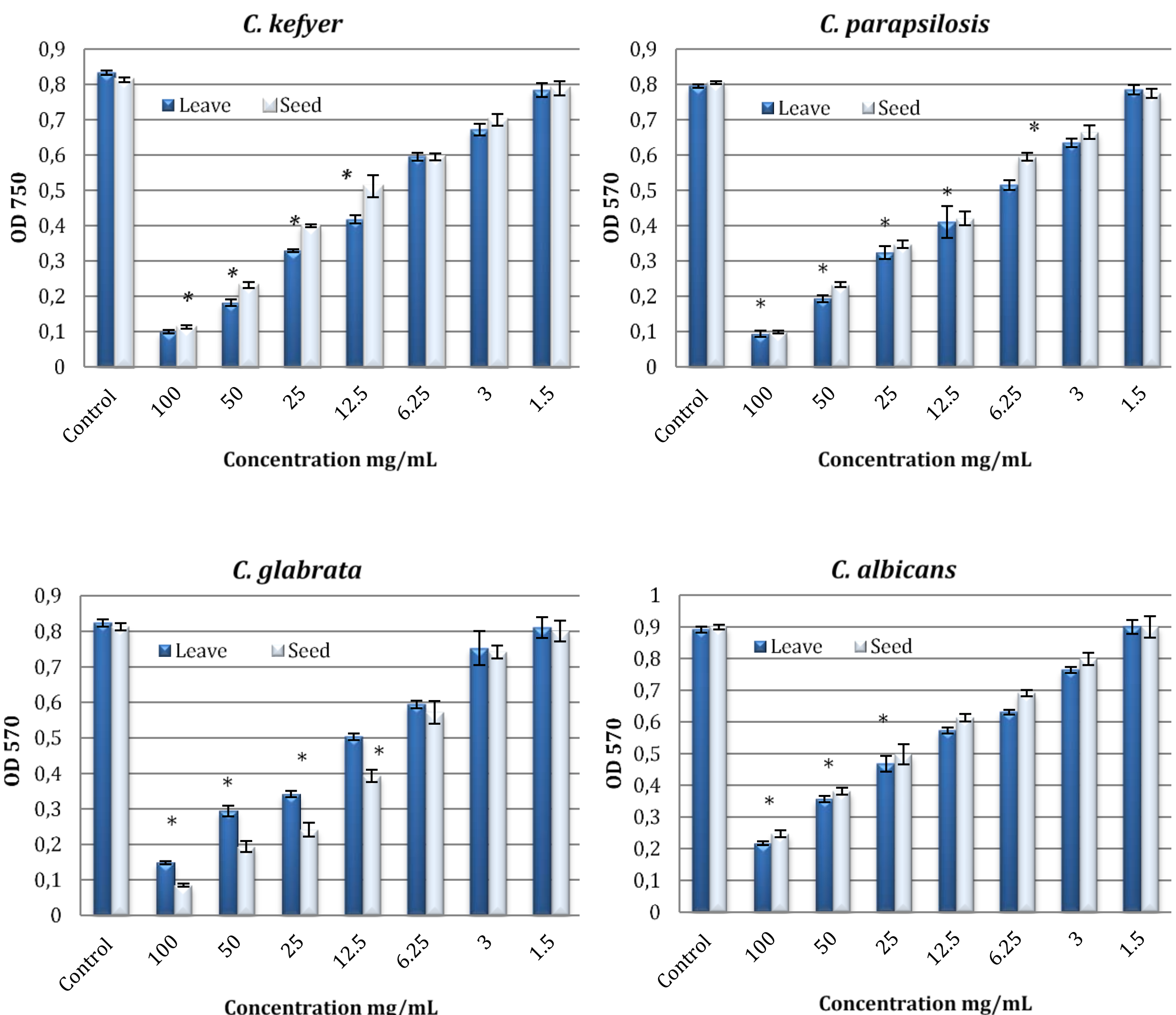


Figure 2. Antifungal activities of Moringa peregrina leave and seed extracts against multi-drug resistant yeast. The values are optical density (OD) read at 570 nm expressed as mean ± standard error of the mean (S.E.M.) of three experiments. Star (*) denote statistically significant differences p ≤0.001, compared with control group (untreated with extract of M. peregrina).

In various countries of Africa and elsewhere, Moringa species have been extensively used to purify water for antiseptic water treatment because of its potent antimicrobial activity **(Rani *et al.*, 2018)**. Innumerable naturally occurring bioactive compounds found in plants, herbs, fruits, vegetables and spices have been shown to possess antimicrobial properties and used as a source to destroy pathogens **(Kumar *et al.*, 2006)**. *Moringa peregrina* is reported to contain a wide range of chemical constituents including flavonoids **(Al-Owaisi *et al.*, 2014)** in the leaves, while arachidonic and linoleic acid, saturated and unsaturated fatty acids and isothiocyanates are present in the seed kernel **(Somali *et al.*, 1984)**.

Our results showed a strong correlation between the MPL and MPS extract concentration and growth inhibition (P<0.001). *Moringa peregrina* extracts revealed a remarkable antimicrobial effect and inhibited the growth of almost all the tested strains in the concentrations ranging from 6 to 100 mg/mL (Tab 1). Among the Gram-negative and gram-positive tested bacteria, *A. baumannii* and *P. aeruginosa* were the most susceptible to *M. peregrina* leaves and seeds extracts (MIC_{50}<6.25) followed by *E. coli* (MIC_{50}≤6.25), S. *aureus* (MIC_{50}≥6.25) and *K. Pneumoniae* (MIC_{50}≤12.5). Results are shown in the Figure 1 and Table 1.

In recent years, the use of natural compounds that are derived from microbials, animals or plants have been shown to possess various antimicrobial activities **(Gyawali *et al.*, 2014; Moloney, 2016)**. The reported phytochemical components in this species include flavonoids, flavanol glycosides, rutin, quercetin, apegenin, glucosinolate and isothiocyanate, phenolic acid, β-sistosterols, alkaloids (lupeol), besides arachidonic and linoleic acids **(Rani *et al.*, 2018)**.

In general, the antibacterial activity of an agent, whether synthetic or natural, is largely attributed to a couple of mechanisms; by hampering chemically the synthesis and/or functions of essential ingredients of the pathogen. Secondly, by preventing the usual mechanisms of antibacterial resistance **(Khameneh *et al.*, 2016; Shakeri *et al.*, 2018)**. The main

mechanism of antibacterial action of these substances is by bacterial protein biosynthesis (by inhibition of protein synthesis) **(Walsh, 2000)**. Thirdly, the biosynthesis of bacterial cell walls as the cell wall layer poses as a valid target for antibacterial agents that consequently weaken the cell wall **(Schneider *et al.*, 2010)**. Furthermore, the inhibition of nucleic acid synthesis, as the enzyme of DNA gyrase is essential for synthesis, repair, replication and transcription process and considered as a suitable target for antibacterial agents. The gyrase enzyme is responsible for coiling and uncoiling of bacterial DNA and replication of DNA (**Maxwell, 1997)**. On the other hand, antifungal potential of the *M. peregrina* among tested fungi strains seem to be high on *C. albicans* (MIC_{50}≤12.5mg/mL) whilst, it has weak activity on *F. oxysporum* and *A. falvus* (MIC_{50}≥50mg/ml) (Tab 1). The results of antimicrobial activity obtained with the extracts of leaves and seeds of *Moringa peregrina* is in accordance of earlier studies **(Majali *et al.*, 2015; Hajar and Gumgumjee, 2014; Saleh *et al.*, 2017; El-Awady *et al.*, 2015; Alrayes *et al.*, 2019)**. The results are tabulated in Table 1. The obtained potent antibacterial and antifungal activity of the moringa leaf and seed extract is due to the presence of an array of bioactive molecules that play an important role in enhancing antibiotic activity against resistant pathogens through diverse mechanisms **(Farhadi *et al.*, 2019; Mansour *et al.*, 2019; Muhuha *et al.*, 2018)**. The bioactive constituents of MP are regarded as potent antioxidants besides their multiple pharmacological activities **(Dehshahri *et al.*, 2012)**.

Table 1. Inhibitory activity of *Moringa peregrina* ethanol extracts of leaves and seeds on the growth and survival of multi-drug resistant pathogens at different extract concentrations ranged from 1.5 -100 mg/mL

		% Inhibition [a]						
Pathogen	**Extracts**	100	50	25	12.5	6.25	3	1.5
Klebsiella pneumoniae	Leaves	94.29	89.63	77.13	58.33	42.04	28.63	1.88
	Seeds	88.67	78.13	63.38	49.33	16.54	8.63	1.37
Acinetobacter baumannii	Leaves	91.07	89.18	86.50	78.15	60.15	35.21	19.41
	Seeds	89.57	78.91	69.58	59.31	52.89	38.56	12.48
Pseudomonas aeruginosa	Leaves	94.95	89.29	79.80	69.67	57.96	40.06	18.45
	Seeds	93.25	88.10	75.91	61.73	53.31	34.78	21.84
Escherichia coli	Leaves	91.67	88.58	82.78	64.54	51.68	22.54	1.42
	Seeds	89.61	79.43	63.48	55.24	38.38	14.34	0.87
Staphylococcus aureus	Leaves	94.71	89.82	80.07	71.19	50.41	39.67	13.57
	Seeds	93.67	86.10	78.70	69.53	44.31	35.12	12.33
Candida kefyr	Leaves	88.61	79.43	66.48	55.24	38.38	4.34	0.27
	Seeds	86.67	74.58	59.78	48.54	39.68	21.54	9.09
Candida parapsilosis	Leaves	81.51	72.36	64.25	50.77	41.45	25.75	6.31
	Seeds	84.71	70.90	59.07	50.19	38.41	22.67	9.57
Candida albicans	Leaves	87.67	79.10	71.70	51.53	38.31	25.12	3.33
	Seeds	88.83	81.09	72.38	57.33	39.42	19.63	8.88
Candida glabrata	Leaves	80.46	70.64	61.40	50.28	37.20	15.54	1.19
	Seeds	85.91	71.15	62.78	54.17	42.53	24.61	2.47
Aspergillus flavus	Leaves	63.97	51.58	43.97	41.18	20.22	9.89	1.73
	Seeds	65.23	54.67	41.21	32.78	22.53	12.95	0.84
Fusarium oxysporum.	Leaves	63.87	51.58	42.07	31.48	23.25	11.71	1.31
	Seeds	62.25	60.91	41.04	34.17	21.96	13.01	3.25

[a] Inhibition (100%): [(Initial control absorbance - final absorbance) / (Initial control absorbance)] x 100.

5. Conclusion

These results support the use of MP in Arab traditional medicine as natural antimicrobial agents. Additionally, the use of such naturally occurring phytochemical components can be used adjacent with synthetic antibiotics to combat bacterial resistance and to enhance the antibacterial potential. Further studies are recommended on isolation and purification of novel antimicrobial molecules to treat the infections caused by microbes.

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Declaration of interest

The authors declare that they have no competing interest.

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