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NOCTURNAL ENURESIS, ANTIOXIDANT AND ANTIMICROBIAL ACTIVITIES OF *PANDANUS ODORATISSIMUS L.* PEDUNCLE

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ABSTRACT: Nocturnal Enuresis treatment activity in rabbits by measuring the changes in urine volume and concentrations of electrolytes as well as bladder capacities. In addition, antioxidant activity was measured using free radical scavenging (DPPH method) and antimicrobial activity against human bacterial pathogens using agar diffusion technique. *Pandanus Odoratissimus L.* peduncle showed no toxic effect with a predicted lethal dose > 16g/kg. The findings indicate the peduncle extracts increase the capacity of the bladder which is an indicator of muscle relaxation with no direct effect on the muscle of dissected bladders. The methanolic extract of the peduncle decrease the urine volume both at 24 hrs and as prescribed traditionally in Yemen (for 3 days) with no effect on its electrolytes. In addition, antioxidant activity of the methanolic peduncle extract was evident by the 83% inhibition of DPPH with different concentrations of the extracts as compared with Vitamin C. In contrast, Peduncle extracts showed no antimicrobial activity against five standard microbial strains (*Micrococcus Lit*, *Staphylococcus aureus*, *Escherichia coli*, *Salmonella enteritidis* and *Candida albicans*). In conclusion, the methanolic peduncle extract of *Pandanus Odoratissimus L.* shows potential Nnocturnal enuresis and antioxidant activities and lacks the antimicrobial activity.

INTRODUCTION: *Pandanus odoratissimus L.*, a shrub, belongs to the family Pandanaceae found along the coast of India and in Andaman Islands Western Malaysia, Sri Lanka¹. Traditionally the parts used from *Pandanus* plant are leaves, roots, male flowers, seeds and fruits². The leaves have been used for the treatment of skin diseases, leprosy, scabies and syphilis as well as remedy for flu, asthma, boils, flatulent colic and morning sickness¹.

Also, it is believed to have aphrodisiac properties and reported to be very efficient for treating jaundice, rheumatism, female sterility and for abnormal menstrual bleeding, whereas dried leaves used to facilitate wound healing^{3, 4}. Traditionally the roots and flowers of *Pandanus Odoratissimus L.* act as an abortifacient and decoction of roots believed to have aphrodisiac and cardi tonic properties as well as being used for diabetes, haemorrhoids and as anti-diuretic³.

The juice squeezed from the aerial root is used in infants with jaundice, restlessness, colic and oral thrush⁴. The oil extracted from flowering tops of *Pandanus Odoratissimus L.* is used to treat earaches and otorrhoea; whereas the anther of male flowers is used in earache, headache and diseases of blood⁵.

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Preliminary phytochemical screening of crude leaf extract of *Pandanus Odoratissimus L.* showed the presence of alkaloids, saponins, flavonoids, steroids, vitamin C, provitamin A carotenoids, carbohydrate and monosaccharides⁶⁻⁹. Chemical component analysis of the root parts led to the isolation of two phenolic compounds, four lignan type compounds plus a new benzofuran derivative which showed strong antioxidative activities¹⁰. Moreover, the leaves of are a natural antioxidant and *Pandanus* extracts are capable of retarding oxidation¹¹.

The hydro-alcoholic, petroleum ether and chloroform extracts of the leaves also showed moderate activity against the gram-positive bacteria⁹. Recently, *Pandanus Odoratissimus L.* (whole plant) exhibited significant antitumor and antioxidant activities in Ehrlich Ascites Carcinoma bearing mice¹²; and its leaves were reported to contain some active principles which possess potential CNS-depressant action in Swiss albino mice¹³. The aqueous extracts of *Pandanus Fascicularis (Odoratissimus)* were demonstrated to possess analgesic activity which is comparable to that of codeine and aspirin and this favours its use in rheumatism and rheumatoid arthritis in traditional medicine¹⁴.

Moreover, methanolic and aqueous extracts of *Pandanus Fascicularis (Odoratissimus) L.* showed significant anti-inflammatory activity in several rodent models by carrageenan induced paw edema, albumin induced plantar edema, acetic acid induced vascular permeability and castor induced diarrhea¹⁵. Alcoholic and aqueous extracts of its roots have been reported to possess anti-hyperglycemic activity in alloxan-induced diabetic rats² as well as antioxidant activity⁸. Recently, the ethanolic extract of prop roots of *Pandanus Fascicularis* was suggested to have beneficial effects as anti-diuretic activity for treatment of polyuria in diabetes insipidus¹¹.

Nocturnal enuresis, otherwise known as night-time incontinence, is a common condition that can cause substantial psychological distress in children and is defined as night time bedwetting in children five years of age or older¹⁶. The prevalence of bedwetting (≥ 2 nights per week) was 8% at 9.5 years¹⁷.

There are three commonly proposed mechanisms to bedwetting include excessive nocturnal urine production, bladder over activity and a failure to awaken in response to bladder sensations. Excessive nocturnal urine production in some children is based on abnormal nocturnal plasma vasopressin release¹⁸. Children with nocturnal enuresis may, however, have sleep disruption. Sleep disruption may result in a loss of the physiologic inhibitory signals to the bladder seen in animal studies¹⁹. This may also be the mechanism behind nocturnal enuresis in children with obstructive sleep apnea²⁰.

Commonly accepted treatments include the bed alarm, desmopressin and tricyclic antidepressants. Desmopressin (synthetic vasopressin) acts on the renal collecting duct and distal tubules to enhance reabsorption of water and is used to treat excessive nocturnal urine production. Tricyclic antidepressants are thought to act via the brainstem through their noradrenergic action; whereby 20% of children who took a tricyclic antidepressant became dry²¹.

Moreover, cardiotoxic and hepatotoxic effects are associated with a tricyclic overdose, and minor adverse effects include postural hypotension, dry mouth, constipation, tachycardia, nausea, lethargy and insomnia. Anti-Cholinergics Drugs act by blocking the parasympathetic nerves that control voiding or by exerting a direct spasmolytic effect on the detrusor muscle of the bladder; thus, targeting bladder dysfunction rather than imbalance between nocturnal urine production and bladder capacity²².

The present study is aimed to evaluate the potential pharmacological activity of both fresh and extracts of *Pandanus Odoratissimus L.* Peduncle in the treatment of Nocturnal Enuresis *in vitro* and *in vivo* as well as its antioxidant and antimicrobial activities.

The *in vitro* study evaluates the relaxant activity of the peduncle extracts on the bladder capacity and the detrusor muscle of such bladders' wall of rabbits, and the *in vivo* evaluates the urine volume at 5 and 24 hrs as well as the electrolytes concentration in urine and blood compared with Desmopressin.

MATERIALS AND METHODS:

Sample collection: The peduncle of the flowers of *Pandanus Odoratissimus L.* were collected from Surdud Valley in Hodaidah, Yemen during flowering stage (April-May 2012). The taxonomy and identification of the plant was confirmed by the Department of Botany, Faculty of Science, Sana'a University, Yemen. The sample was dried by separating the used part (Peduncle) from the whole plant then left to dry away from sunlight and moisture. The dried sample was weighed before and after grinding using Apx-100, Denver instruments electric balance.

Extraction of *Pandanus Odoratissimus L.*: Dried powdered peduncle of *Pandanus Odoratissimus L.* (841.9 g) were exhausted several times by maceration in 4 L methanol (99.9%) for several weeks, and the combined methanolic extracts were evaporated under reduced pressure using rotary evaporator.

One hundred gram of semi-solid residue of the total extract was then diluted by methanol and distilled water, to which 150 ml of petroleum ether was added in a separating funnel, shook and left to stand for a while prior to the collection of the petroleum ether fraction in a beaker. This process was repeated twice. The previous steps were then repeated with different solvents each time, beginning with chloroform, ethyl acetate, methanol and water respectively (Elutropic System). The obtained fractions were concentrated via rotary evaporator.

Preliminary Phytochemical Screening: A preliminary phytochemical analysis of plant extracts was carried out using Thin Layer Chromatography (TLC) plates coated with silica gel 60 F254 for TLC. The mobile phase ethyl acetate: methanol: water (30:5:4) was added to the chromatography-tank and left for a time to saturate the tank; micro-drops of the concentrated solutions of the fractions obtained (crude, petroleum ether, chloroform, ethyl acetate, methanol and aqueous extracts) were spotted on pre-coated Silica gel. The chromatogram after complete development was air dried and visualized with different chemical reagents to detect the presence of flavonoids, phenols, tannins, alkaloids, steroids, amino acids, saponins, and triterpenes glycosides.

Experimental animals: Rabbits with an average weight of 1000g were used and the animals were fed with standard animal feed and water. Animals were acclimatized to the laboratory conditions for 3 weeks prior to experimentation. All experiments carried out were approved by the Institutional Ethical Committee, Faculty of Medicine and Health Sciences, Sana'a University.

Acute toxicity study: For determination of the dose of LD50, 10 rabbits were used and randomly assigned to control or treatment groups (2 animals per group). Animals were deprived of food but given water 16-18 hours prior to dosing. Fresh and extracts at a doses of 10 and 16 g/kg were then given orally to test groups, while the control group received water at the same volume. Body weight, signs of toxicity (general behaviour, motor activities, aggressiveness, reaction to noise, reaction to pinch, state of tail and state of excrement) and mortality were observed after administration at the third hour on the first day, and throughout the following 48 hours²³.

In vitro determination of bladder relaxation: Isolated rabbit bladders were inserted into normal saline in ice bath immediately after dissection. Bladders were grouped in two and treated with 1 ml Neostigmine followed by either 1 ml Mydoclam or any of the three different extracts (crude, aqueous and methanolic) of the peduncle at three different concentrations (0.5, 1 and 2g) and the relaxation and contraction of the bladders were observed by a monograph.

In vivo determination of urine volume: Two different sets of animals were used to study the acute effect over a 24 hr period and over 3 days as traditionally used (twice daily for three days). Each set contained 24 rabbits (3 animals per group) assigned to control or treatment [exposed to either demsopressin (as standard) or the three different extracts (crude, methanolic and aqueous) of the peduncle at two different concentrations (250 and 500 mg/kg body weight)].

Diuresis was induced in all animals by furosemide (20mg/kg). Urine volume (measured at 5 and 24 hrs of treatment) as well as urine and blood electrolytes were determined and expressed in mMol/L¹¹.

Rabbits were then dissected and the bladder capacity was determined by measuring the urine volume via recording the elevation or reduction of water level in pipettes that were tightly fixed into the bladders.

Determination of the Antioxidant activity (DPPH Method): The DPPH (2,2-Diphenyl-1-picrylhydrazyl) radical scavenging activity was determined by a standard method. Stock solutions of the tested compounds with concentrations of 0.1, 0.2, 0.4, 0.8 and 1.6 mM were prepared in absolute methanol, and a stock solution of the DPPH with a concentration of 0.6 mM was prepared in the same solvent. From each stock solution 100 µl aliquots was transferred into a 96-well plate (Greiner Bio-One) containing 100 µl of the DPPH stock solution to produce a test sample of 200 µl final volume containing 300 µM DPPH as a final concentration and 200, 400, and 800 µM final concentrations of the test compounds.

Ascorbic acid was used as the positive control and the negative control was prepared as 300 µM DPPH in pure methanol. These mixtures were incubated for 30 minutes at room temperature, and the absorbance of these mixtures was measured using a multi-well scanning spectrophotometer (Wallac, Victor, 1420 multilaboratory counter) at 517 nm. All experiments were performed in triplicate. The inhibition percentage was calculated using the following formula:

$$\text{DPPH scavenging activity (\%)} = 1 - \frac{\text{Absorbance}_{\text{sample}}}{\text{Absorbance}_{\text{control}}} \times 100$$

Determination of the Anti-microbial activity (Agar disc diffusion method): The anti-microbial

activity of the various extracts was done on Mueller Hinton agar (MHA) using agar disc diffusion method. Five microbial strains were selected on the basis of their clinical importance in causing diseases in humans [two gram-positive bacteria (*Staphylococcus aureus* and *Micrococcus Lit*); two gram-negative bacteria (*Escherichia coli* and *Salmonella enteritidis*); and one yeast (*Candida albicans*)] for evaluation of the antimicrobial activity. A swab was dipped into the broth culture of the organism and gently squeezed against the inside of the tube to remove excess fluid. The swab was then used to streak a Mueller-Hinton agar plate or a nutrient agar plate for a lawn of growth.

The plates were allowed to dry for about 5 minutes prior to placing the extracts and antibiotic disks on the surface of the agar using flame sterilized forceps. The plates were inverted and incubate for 24 hours at 37° C and the diameter of the zone of inhibition (if present) was measured for each extract and antibiotic used. All experiments were performed in triplicate.

RESULTS AND DISCUSSION: The weight of extracts obtained by fractionation was: petroleum ether (5.05 g), chloroform (1.78 g), ethyl acetate (4.14 g), methanolic (38.29 g), and aqueous (30.25 g). Preliminary chemical examination of the different fractions (**Table 1**) reveal the presence of: flavonoids and phenolic compounds in methanolic, ethyl acetate and aqueous extracts; alkaloids in petroleum ether and chloroform extracts; steroids and amino acids in crude, chloroform, ethyl acetate and methanolic extracts; as well as triterpenes glycosides in crude, ethyl acetate, methanolic and aqueous extracts. Persisting foam was formed in all of the extracts suggesting the presence of saponins.

TABLE 1: PRELIMINARY PHYTOCHEMICAL SCREENING

Chemical constituent	Chemical tests	Extracts					
		P	Ch	E	M	A	C
Flavonoids	AlCl ₃	-	-	+	+	+	+
	KOH	-	-	+	+	+	+
Phenols + Tannins	FeCl ₃	-	-	+	+	+	+
	NH ₃ vapor	-	-	+	+	+	+
Alkaloids	Dragendorff's reagent	+	+	-	-	-	-
Steroids + Amino acids	Vanillin reagent	-	+	+	+	-	+
Steroids + Triterpene glucosides	Acetic anhydride, H ₂ SO ₄	-	-	+	+	+	+
Saponins	Froth test	+	+	+	+	+	+

(+) Present, (-) absent, (P) Petroleum ether, (Ch) Chloroform, (E) Ethyl acetate, (M) Methanolic, (A) Aqueous, (C) Crude extract

The observed behavioral changes and toxicological signs (**Table 2**) show the motor activity to be reduced and profoundly reduced in the groups exposed to the higher concentration of 16g/kg of fresh and crude extract, respectively as compared to the control group. The aggressiveness and reaction to noise were profoundly reduced in those exposed to the 16g/kg crude extract. No effect was observed with regard to the following parameters: reaction to pinch, state of tail, state of excrement, clonic convulsion and salivation. Moreover, no mortality was observed within 48 hours after oral

administration of both the fresh plant and the crude extract. The oral acute toxicity study did not reveal any grossly toxicological signs with the lethal dose (LD50) being predicted to be greater than 16g/kg (LD50 > 16g/kg), suggesting that the *Pandanus Odoratissimus L.* peduncle is practically non-toxic at the concentrations used in this study and have a reasonable margin of safety. Previous studies have reported the methanolic leaf extract of *Pandanus Odoratissimus L.* to be non-toxic with an LD50 > 2g/kg¹³.

TABLE 2: EFFECT OF FRESH AND CRUDE EXTRACT OF PEDUNCLE ON TOXICITY SIGNS

Parameters	Groups and behavior of animals				
	Control	Fresh		Crude Extract	
		10g/kg	16g/kg	10g/kg	16g/kg
Motor activity	N	N	-	N	--
Aggressiveness	N	N	N	N	--
Reaction to noise	N	N	N	N	--
Reaction to pinch	N	N	N	N	N
State of tail	N	N	N	N	N
State of excrement	N	N	N	N	N
Clonic convulsion	N	N	N	N	N
Salivation	N	N	N	N	N
Mortality (within 48 hrs)	NM	NM	NM	NM	NM

(N) Normal, (-) Reduced, (- -) profoundly reduced, (NM) No Mortality

Table 3 highlights the results of the pharmacological study for the relaxant activity of plant extracts on the detrusor muscle of bladders' wall. Muscle contraction was evident in the neostigmine group. Unlike the observed relaxation

in the mydocalm treated group, addition of the different extracts of *Pandanus Odoratissimus L.* peduncle (crude, methanolic and aqueous) did not result in relaxation of the detrusor muscles following the contraction with neostigmine.

TABLE 3: IN VITRO EFFECT OF DIFFERENT PEDUNCLE EXTRACTS ON THE DETRUSOR MUSCLE OF BLADDERS' WALL

Groups	Drugs			Extracts	
	Neostigmine	Mydocalm	Crude	Aqueous	Methanolic
1	+	*	*	*	*
2	*	#	*	*	*
3	+	-	*	*	*
4	+	*	*	*	#
5	+	*	*	*	#
6	+	*	*	*	#
7	+	*	*	#	*
8	+	*	*	#	*
9	+	*	*	#	*
10	+	*	#	*	*
11	+	*	#	*	*
12	+	*	#	*	*

(+) Contraction, (-) Relaxation, (#) No change, (*) No addition

Moreover, the effect of the peduncle extracts on urine volume (at 5 and 24 hrs) and electrolytes concentration in urine and blood (**Table 4**) showed

the urine volume in the desmopressin group to be lower by 63.1% and 45.9% with respect to the control group at 5 and 24 hrs respectively.

TABLE 4: EFFECT OF DIFFERENT PEDUNCLE EXTRACTS ON URINE VOLUME, URINE AND BLOOD ELECTROLYTES AT 24 HOURS

Group	Dose (mg)	Urine volume		Electrolyte excretion (mM/L) /5 hr			Blood Electrolytes		
		ml /5 hrs	ml /24 hrs	Na ⁺	K ⁺	Cl ⁻	Na ⁺ (130-150 mM)	K ⁺ (3.6-7.5 mM)	Cl ⁻ (85-120 mM)
Control		111	196	78	79.9	78	134	5.4	87
Desmopressin	0.0093mg	41	106	56	36	79	137.5	7	99
Crude	250mg	112	198	77	61	96	136	5.6	87.5
	500mg	108	181	63	70	66	135	6.3	90.5
Aqueous	250mg	110	220	77	61	69	136	5.5	87
	500mg	105	215	78	63	90	137	7	88
Methanolic	250mg	110	152	76	72	83	136.5	6.5	89
	500mg	115	148	74	78	112	137	6.7	91

In contrast, the urine volumes at 5 and 24 hrs were not different in any of the groups exposed to the *Pandanus Odoratissimus L.* peduncle extracts as compared to the control group; with the exception of the methanolic extract showing a moderate decrease at the 24 hrs collection of 22.4% and 24.5% respectively. The electrolyte concentrations in urine and blood, however, were not affected by these extracts. On examining the effect of the Yemeni traditional use of *Pandanus Odoratissimus*

L. peduncle extracts on the urine volume and blood electrolytes (**Table 5**), the desmopressin group showed a lower urine volume (21.4%) with respect to the control group. Similar effect was observed in the group exposed to the methanolic extract (19-24.6%). A moderate decrease in the urine volume was also observed in the group exposed to the crude extract (10-15%); with the least effect in the aqueous extract group (6-8.7%). In contrast, no effect was observed on the blood electrolytes.

TABLE 5: EFFECT OF DIFFERENT PEDUNCLE EXTRACTS ON URINE VOLUME AND BLOOD ELECTROLYTES AS TRADITIONALLY USED (after 3 days)

Group	Dose (/kg)	Urine volume (%)	Blood Electrolytes		
			Na ⁺ (130-150 mM)	K ⁺ (3.6-7.5 mM)	Cl ⁻ (85-120 mM)
Control		101.5	136	5.2	100
Desmopressin	0.0093mg	79.8	134	5.3	96.5
Crude	250mg	91.3	138	5.1	97
	500mg	86.1	136	5.3	98.5
Aqueous	250mg	95	139	5.5	100
	500mg	92.7	138	5.5	99
Methanolic	250mg	81.8	138	5.1	102
	500mg	76.5	135	4.9	97

In this study, the activity of *Pandanus Odoratissimus L.* peduncle in the treatment of Nocturnal Enuresis was proved to be successful by using different experiments. The findings indicate that peduncle extracts have no *in vitro* direct effect on the muscle of dissected bladders. However, *in vivo* experiment showed increase in the capacity of the bladder which is an indicator of muscle relaxation. Both the acute and traditional use of *Pandanus Odoratissimus L.* peduncle moderately decreased the urine volume and the bladder capacity (urine volume) of dissected rabbits (**Table 6**) exposed to the methanolic extract showed a moderate increase (38-44%) as compared to the control group. On the other hand, no change was observed in either the crude or aqueous extracts.

Recently, the ethanol extract of prop roots of *P. fascicularis* was suggested to possess an effective anti-diuretic activity, which supports the claim that the plant can be used as anti-diuretic ¹¹.

The authors also provided basis for the traditional use of *P. fascicularis* Lam. in treatment of diabetes insipidus.

Table 7 shows the methanolic extract of *Pandanus Odoratissimus L.* peduncle to have an overall high antioxidant activity comparable to ascorbic acid (positive control), with highest antioxidant activity (86%) at concentration 800 µg/ml, followed by (84% and 83%) at 400 µg/ml and 200 µg/ml, respectively.

TABLE 6: BLADDER CAPACITY (URINE VOLUME) OF BLADDERS FROM DISSECTED RABBITS EXPOSED TO DIFFERENT PEDUNCLE EXTRACTS AT 24 HRS.

Group		Capacity of bladder (ml)
Control		32
Desmopressin	0.00886mg	58
	250mg	33
Crude	500mg	35
	250mg	33
Aqueous	500mg	35
	250mg	44
Methanolic	500mg	46

TABLE 7: ANTIOXIDANT ACTIVITY OF METHANOLIC PEDUNCLE EXTRACT OF PANDANUS ODORATISSIMUS L.

Concentration	<i>Pandanus Odoratissimus. L</i>	Vitamin C
200 µg/ml	0.201 (83%)	0.149 (88%)
400 µg/ml	0.173 (84%)	0.144 (88%)
800 µg/ml	0.187 (86%)	0.151 (88%)

The results of this study infer that the methanol peduncle extract of *Pandanus Odoratissimus L.* reduces the radical to the corresponding hydrazine when it reacts with the hydrogen ions released from the samples suggesting the presence of some active constituents in the methanol peduncle extract. This corresponds with the presence of polyphenolics in the TLC screening of the extracts, suggesting that the antioxidant capacity of the plant extracts is due to a great extent to their polyphenols.

Hence this plant can be considered as potential sources of bioactive compounds acting as natural antioxidant. Similar results have shown the methanolic and aqueous root extract of *Pandanus Odoratissimus L.* to have high antioxidant potential as observed in both DPPH scavenging assay and reducing capacity⁸. This corresponded with a positive correlation with the phenolics and flavonoid contents observed in the root extracts. These findings also indicate that polyphenolic components may have antioxidant potential, as reported in other medicinal plants.

Moreover, the methanolic leaf extract of *Pandanus Odoratissimus L.* has also been reported to possess moderate activity of antioxidant enzymes such as lipid peroxidase, catalase, superoxide dismutase and glutathione^{24, 25}.

The antimicrobial activity of the different extracts of *Pandanus Odoratissimus L.* peduncle (crude, petroleum ether, chloroform, ethyl acetate, methanolic, and aqueous) showed no significant antimicrobial activity against the five standard microbial strains that were selected for this study: gram-positive (*Staphylococcus aureus*, *Micrococcus Lit*), gram-negative (*Escherichia coli* and *Salmonella enteritidis*) bacteria and yeast (*Candida albicans*) as measured by the inhibition zone [Data not shown]. Unlike the peduncle extracts, the hydroalcoholic, chloroform and petroleum ether extracts of the leaves exhibited effective inhibition zones against gram-positive bacterial strains and being ineffective against gram-negative bacteria and yeast⁹.

The authors attributed the good antimicrobial activity in the leaves to the presence of alkaloids and flavonoids which were showed in hydroalcoholic extract by phytochemical screening⁹. Other results have shown the petroleum ether, chloroform and methanolic extracts of the *Pandanus Odoratissimus L.* (whole plant) to possess a potential broad spectrum antibacterial activity against both gram-positive and gram-negative bacteria, such as *Escherichia coli*, *Staphylococcus aureus*, *Proteus ulgaris*, *Pseudomonas aeruginosa*, *Klebsiella pneumoniae* and fungi like *Aspergillus niger*, *Neurospora curcus*⁷.

The discrepancy observed may be due to the different nature of bioactive compound from one part to another of inflorescent of *Pandanus Odoratissimus L.*

CONCLUSION: In conclusion, from the above results, it can be suggested that the methanolic peduncle extract of *Pandanus Odoratissimus L.* has a potential Nocturnal enuresis and antioxidant activities and lacks the antimicrobial activity; which supports the traditional claim that the plant can be used for the treatment of Nocturnal enuresis.

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