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In Vitro Antimicrobial Properties of *Uroteuthis edulis* (Squid) Extract Against Uropathogenic Bacteria

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ABSTRACT

Urinary tract infections (UTIs) are a major worldwide health issue, especially among women, and Uropathogenic *Escherichia coli (E. coli)* is the most common causal agent. However, the effectiveness of traditional therapies, such as nitrofurantoin, is hampered by antibiotic resistance, exacerbated by premature medication discontinuation. This study examines the possibility of *Uroteuthis edulis* (Squid) ink extract as an alternative therapy option for UTIs. The study used quantitative experimental approaches to establish squid ink's potential antibacterial activity against *Escherichia coli and Staphylococcus saprophyticus*. The findings highlight the untapped potential of marine-derived compounds in addressing the growing challenge of antibiotic resistance in UTI treatment paradigms.

The Antimicrobial Susceptibility Testing (AST) is composed of Minimum Inhibitory Concentration (MIC) using the Microboth dilution method and the Minimum Bactericidal Concentration (MBC) using the Agar disk diffusion method. It is followed by a two-fold dilution of decreasing concentration of 1000µ/mL. Results of MIC included promising results of concentrations at 62.5 µg/mL for gram-positive *S.saprophyticus* while 250 µg/mL for gram-negative *Escherichia coli*. Results of MBC included concentrations of 250µg/mL for *S.saprophyticus* and 500µg/mL *for Escherichia coli*. *An independent T-test was used to determine the significant difference between the* MIC and MBC *of Urotheuthis edulis to the positive control for Staphylococcus saprophyticus and Escherichia coli.* Findings from the independent t-test reveal that the p-value implies a significant difference between the Minimum Inhibitory Concentration of both *S. saprophyticus* (p-value of 0.000 < 0.05) and *E. coli* (p-value< 0.05). Similarly, the MBC findings have shown significant differences between both *S. saprophyticus* (p-value < 0.05) and *E. coli* (p-value <0.05) to the positive control, nitrofurantoin.

Therefore, the ink extracts exhibited inhibitory and bactericidal effects on both the presented bacterial isolates, *Escherichia coli* and *Staphylococcus saprophyticus* and have a promising potential for in vivo studies. These in vitro efficacy, however, need further work to validate in vivo.

Keywords: *Urinary tract infections, Antibiotic resistance, Squid ink extract, Alternative therapy, Escherichia coli, Marine-derived compounds*

1. INTRODUCTION

Urinary tract infections (UTIs) are a pervasive global health concern, affecting millions annually, particularly women[1]. These infections are primarily caused by various microorganisms, with the gram-negative *Escherichia coli* being the most common and significant pathogen[2]. Immediately following E*. Coli*, the gram-positive *S. saprophyticus,* is considered to be the second most common causative agent of uncomplicated UTIs in women[3].

Antibiotics such as ampicillin, cefalotin, ciprofloxacin and nitrofurantoin have been central to treatment strategies[4]. However, the increasing prevalence of antimicrobial resistance (AMR) poses a formidable challenge to the effectiveness of these traditional therapies. AMR not only complicates the treatment of UTIs but also contributes significantly to global morbidity and mortality, with a reported 1.27 million deaths in 2019, highlighting an urgent need for coordinated global efforts to manage and mitigate its impact[5].

In the ongoing battle against antibiotic resistance, researchers have found that squid ink's potential antibacterial activity against environments rich in biodiversity has emerged as a promising reservoir for bioactive compounds with significant antimicrobial properties [6]. This pursuit is especially pertinent given the mounting resistance to conventional antibiotics among pathogenic microorganisms. Among the marine species under investigation is *Uroteuthis edulis*, commonly known as squid, which harbours alkaloids with demonstrated antibacterial effectiveness. These alkaloids play a pivotal role in antimicrobial defence by destabilising the cytoplasmic membrane of bacterial cells, thereby interfering with vital cellular functions[7]. This research highlights the critical need for and potential of marine-derived substances in developing novel antibacterial therapies.

2. METHODOLOGY

Uroteuthis edulis squid samples were ethically sourced from Trabaho market, Manila. Upon collection, samples were immediately transported in an ice chest to maintain a temperature of 4°C[8], ensuring preservation and minimising bacterial contamination during transit. The National Fisheries and Research Development verified the squid to be *Uroteuthis edulis* through morphological examination and confirmed by genetic analysis. Suppliers from MAC Tycoon Marketing, located in Bambang, Sta, acquired the nitrofurantoin. Cruz, Manila.

The clinical isolates of *E.coli* and *S. saprophyticus* were acquired from Medilinx, Quezon City, pre-made using 0.5 McFarland standard as a clinical isolate. The selection criteria were designed to guarantee that the experimental setup included a representative sample of the target pathogens.

The experiment was conducted at Far Eastern University, Manila, within dedicated academic-centred laboratory facilities designed to accommodate experimental medical trials, thus ensuring the safety and precision required for such research endeavours.

In performing the extraction of squid ink, the squids were washed with tap water and then distilled water.

Afterwards, position the squid posteroventrally with sterile scissors to obtain the ink glands. The gland was squeezed directly into the amber bottle, obtaining 5ml squid ink. The squid ink was macerated at a 1:3 ratio of squid ink, and 30 ml of hexane was combined [9]. The mixture is incubated for seven days at 4 C. It is then centrifuged at 15,000 rpm for 30 minutes to acquire the supernatant[10].

Figure 1. Extraction Phase

The supernatant is then subjected to vacuum evaporation using Rotavap at 67°C for 1 hour. The solvent boils at a lower temperature than usual due to the apparatus' decreased pressure, and the liquid surface area and evaporation rate are increased when the flask is rotated[11]. The evaporation process was carried out below hexane's boiling point (below 68.7℃) until all the solvents had been withdrawn [12].

The agar dilution technique was utilised to qualitatively assess the in vitro activity of the squid ink antimicrobial compound and the commercial antibiotic nitrofurantoin against the uropathogenic test bacteria [13]. A 0.5 McFarland standard of ATC *E. coli* and *S. saprophyticus* were prepared,

ensuring that the bacterial count was 1 x 105 CFU/mL. Baltazar's protocols were followed for MIC testing[14]. The broth microdilution method was employed for the Minimum Inhibitory Concentration (MIC) testing, utilising stock solutions of *Uroteuthis edulis* squid extract prepared with DMSO. Serial two-fold dilutions were then performed to create ten concentrations ranging from 1000 ug/mL to 1.95 ug/mL. These dilutions were then carefully arranged in a 96-well plate, with positive dimethylsulfoxide (DMSO) controls and negative control of a 300 µg Nitrofurantoin disk, following a decreasing order from left to right. The MIC was triplicated for each isolate of *E.coli* and *S.saprophyticus* [15]. Afterwards, the MIC is incubated at 37 °C for 24 hours. After 24 hours, the 96-well plate is placed in a Microplate reader at 405 nm for each microplate [16].

Based on the absorbance wells that are less of the negative control, they were subjected to Minimum Bactericidal Concentration (MBC) determination. The MBC testing utilised the Agar Disk Diffusion method, with concentrations clear from the MIC reading applied to filter paper disks containing 15 µL and air-dried before incubation[17].

The disk diffusion assay was done by dipping a sterile cotton swab into an adjusted suspension. To ensure a uniform distribution of inoculum, the cotton swab filled with the bacterial suspension was distributed on the surface of Mueller-Hinton agar and repeated four times. The blank discs containing the squid ink antimicrobial compounds were placed evenly on the Mueller Hinton agar surface, providing sufficient coverage for microorganism inhibition. The petri dish with inoculum and the antimicrobial disc was inverted and incubated at 35 ± 2°C for 18-24 hrs [18].

The resulting zone of inhibition was measured using a calliper, facilitating comparison with the positive and negative controls. Additionally, the squid ink antibiotic disks were appropriately stored in amber bottles at room temperature, away from sunlight, ensuring the preservation of their antibacterial properties.

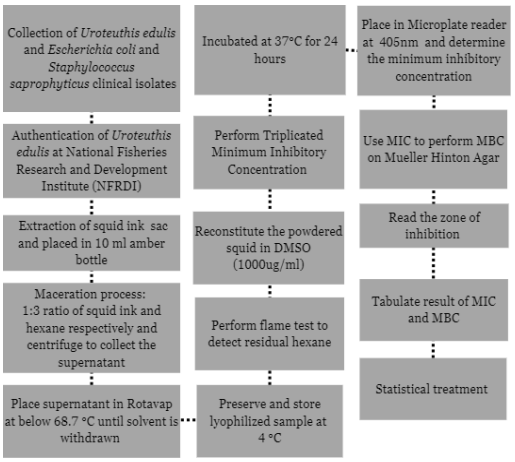

Figure 2. Data Collection Procedure

3. RESULTS AND DISCUSSION

As the study examines the efficacy of the *Uroteuthis edulis* ink as an antimicrobial compound, the researchers have found a significant difference between the *Urotheuthis edulis* extract and nitrofurantoin.

3.1 Phytochemical Testing for Alkaloids

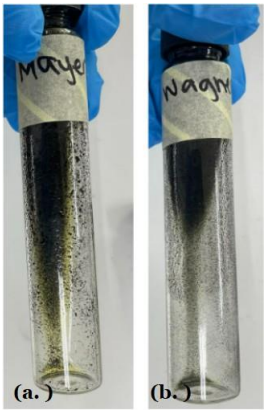

Figure 3. (a)Mayer's and (b)Wagner's test

Parbuntari's method, consisting of Mayer's test and Wagner's test, was used to detect the presence of alkaloids in the squid ink extract[19]. The squid ink extract showed a positive result that exhibited a cream-coloured precipitate for Mayer's test. Wagner's test, as a confirmatory test, later tested positive for a reddish-brown complex. The presence of these alkaloid compounds is highly likely responsible for the antimicrobial properties of squid ink.

To quantitatively assess the antimicrobial properties of the alkaloids present in the squid ink extract, the Minimum Inhibitory Concentration (MIC) assay was used. The MIC assay provided significant data

about the minimum concentration of the squid ink extract required to prevent the growth of specific microbial strains, hence establishing its potential as an antimicrobial agent for uropathogenic bacteria [20].

Table 1. Mean Absorbance of the Microbroth Dilution Method

TRIALS		Mean Absorbance of Concentration for the Crude Solvent Extracts										MIC Value (in µg/mL)		
		1000	500	250	125	62.5	31.25	15.63	7.81	3.91	1.95	+Ve	-Ve	Solvent
Staphylococcus saprophyticus	1	0.173	0.144	0.117	0.178	0.249	0.313	0.347	0.357	0.273	0.2			
	2	0.165	0.118	0.092	0.18	0.283	0.331	0.342	0.325	0.378	0.385			
	3	0.173	0.126	0.102	0.223	0.264	0.326	0.348	0.345	0.373	0.348	0.112	0.278	0.083
MEAN		.170	.129	.104	.194	.265	.323	.346	.342	0.341	0.311			
Escherichia coli	1	0.162	0.146	0.104	0.288	0.286	0.283	0.288	0.256	0.261	0.263			
	2	0.155	0.132	0.181	0.289	0.318	0.102	0.305	0.102	0.246	0.282			
	3	0.17	0.146	0.11	0.268	0.279	0.291	0.29	0.268	0.271	0.235	0.101	0.256	0.003
MEAN		0.162	0.141	0.132	0.282	0.294	0.225	0.294	0.209	0.259	0.26			

This table demonstrates the Minimum concentration of the crude extract in decreasing two-fold dilution. The positive control (+ve) contains 5µL bacterial isolates and 100 µL Mueller Hinton Broth (MHB). Negative control (-ve) contains MHB only. Solvent control contains 5µL bacterial isolates, MHB and 100 µL DMSO. The microplate reader runs on the principle of detecting light signals by specific wavelengths, which means the lower the absorbance, the more turbid the sample is[21]. Thus, the crude extracts' absorbance was compared to the -ve, and the absorbance that was lower than the -ve control meant that the antibiotic was able to inhibit bacterial growth as compared to the +ve control. Concentration 62.5 µg/mL shows the MIC required to prevent the growth of the Staphylococcus saprophyticus. In Escherichia coli, 250 µg/mL is the MIC. This will serve as the base concentration of squid ink extract used in the MBC of *Escherichia coli* and *Staphylococcus saprophyticus*.

Wells 1000, 500, 250, 125 and 62.5 µg/mL for *S. saprophyticus* and 1000, 500, 250µg/mL for *E.coli* were subjected to Agar Disk Diffusion for Minimum Bactericidal Concentration.

Table 2. Mean Inhibition of the Agar Disk Diffusion Method

TRIALS		Mean Zone of Inhibition (in mm)					MBC Value (in µg/mL)	
		1000	500	250	125	62.5		
Staphylococcus saprophyticus	1	18.35	14.15	9.55	0	0	+Ve	-Ve
	2	17.55	15.85	10.35	0	0	22.55	0
	3	17.95	15.25	9.75	0	0	23.65	0
MEAN		17.95	15.08	9.88	0	0	22.85	0
Escherichia coli	1	18.45	12.15	0			24.85	0
	2	17.75	12.75	0			26.15	0
	3	18	13.75	0			26.85	0
MEAN		18.07	12.88	0			25.95	0

The mean inhibition zones, measured in millimetres (mm), were determined for triplicate plates at each concentration of the squid ink extract against *Staphylococcus*

saprophyticus and *Escherichia coli*. Nitrofurantoin disk was employed as the positive control, while DMSO was the negative control.

For *Staphylococcus saprophyticus,* concentrations 1-3 of the squid ink extract exhibited inhibition, whereas only concentrations 1 and 2 demonstrated inhibition against *Escherichia coli*. The MBC for *S. saprophyticus* is 250µg/mL, while 500µg/mL for *E.coli.*

According to the CLSI guidelines 2021, the standard zone of inhibitions for nitrofurantoin against both Escherichia coli and *Staphylococcus saprophyticus* are as follows[22]:

- Susceptible: ≥ 17 mm
- Intermediately susceptible: 15-16 mm
- Resistant: ≤ 14 mm

Comparing these standards to our data, we discovered that the inhibition zones for nitrofurantoin against both bacterial isolates were within the susceptible range.

The findings reveal that the two bacterial strains had differing sensitivity to the squid ink extract, the gram-positive *S. saprophyticus* being more sensitive across a broader range of concentrations than the gram-negative *Escherichia coli*.

Table 3. **Results from the independent T-test for Minimum Inhibitory Concentration (MIC)**

		Significant Difference of the MIC									
			Levene's test for Equality of Variance		T-test for Equality of Means						
			F	Sig	t	df	Sig (2-tailed)	Mean Difference	Std. Error Difference	95% Confidence Interval of the Difference	
										Lower	Upper
MIC	*S. saprophyticus*	Equal variances assumed	5.059	.088	103.345	4	.000	1.01666	.009838	.989353	1.04398
		Equal variances not assumed			103.345	2.00	.000	1.01666	.009838	.974339	1.05899
	E. coli	Equal variances assumed	15.32	.017	45.157	4	.000	1.141333	.024727	1.0722679	1.20999
		Equal variances not assumed			45.157	2.00	.000	1.141333	.024727	1.034940	1.247727

An independent T-test was used to determine whether there is a significant difference between the Minimum Inhibitory Concentration (MIC) of *Urotheuthis edulis* and positive control for *Staphylococcus saprophyticus and Escherichia coli.*

Findings of the independent t-test, as shown in Table 4, reveal that the p-value for Levene's Test for Equality is 0.088; since the p-value > 0.05, the result lies within the first row or equal variance is assumed. The result of the T-test shows that the t-value is 103.345, and the p-value is 0.000. This implies that there is a significant difference between the Minimum Inhibitory Concentration (MIC) of *Urotheuthis Edulis* and positive control for *Staphylococcus saprophyticus* because the p-value < 0.05.

Another finding from the independent t-test, as shown in Table 4, reveals that the p-value for Levene's Test for Equality is 0.017; since the p-value < 0.05, the result lies within the second row or equal variance is not assumed. The result of the T-test shows that the t-value is 46.157, and the p-value is 0.000. This implies a significant difference between the Minimum Inhibitory Concentration (MIC) of *Urotheuthis Edulis* and positive control for *Escherichia coli* because the p-value < 0.05.

Table 4. Results from the independent T-test for Minimum Bactericidal Concentration (MBC)

		Significant Difference of the MBC									
			Levene's test for Equality of Variance		T-test for Equality of Means						
										95% Confidence Interval of the Difference	
			F	Sig	t	df	Sig (2-tailed)	Mean Difference	Std. Error Difference	Lower	Upper
MBC	*S. saprophyticus*	Equal variances assumed	10.316	.033	-35.784	4	.000	-8.601333	.24037	-9.26870	-7.93395
		Equal variances not assumed			-35.784	2.00	.000	1.01666	.24037	-9.26870	-7.56710
	E. coli	Equal variances assumed	65.63	.063	-24.879	4	.000	-11.610333	.466667	-12.9060	-10.3146
		Equal variances not assumed			-24.879	2.00	.002	-11.610333	.466667	-13.6182	-9.60242

An independent T-test was used to determine whether there is a significant difference between the Minimum Bactericidal Concentration (MBC) of *Urotheuthis edulis* and positive control for *Staphylococcus saprophyticus and Escherichia coli.* Findings from the independent t-test, as shown in Table 4, reveal that the p-value for Levene's Test for Equality is 0.033; since the p-value <0.05, the result lies within the second row or equal variance is not assumed. The result of the T-test shows that the t-value is -35.784, and the p-value is 0.001. This implies a significant difference between the Minimum Bactericidal Concentration (MBC) of *Urotheuthis Edulis* and negative control for *Staphylococcus saprophyticus,* the p-value < 0.05.

Another Finding from the independent t-test, as shown in Table 4, is that the p-value for Levene's Test for Equality is 0.063; since the p-value > 0.05, the result lies within the first row or equal variance is assumed. The result of the T-test shows that the t-value is -24.879, and the p-value is 0.000. This implies a significant difference between the Minimum Bactericidal Concentration (MBC) of *Urotheuthis Edulis* and positive control for *Escherichia coli* because the p-value < 0.05.

These results led the researchers to reject the null hypotheses, indicating that the alkaloids found in *Uroteuthis edulis* squid ink sac have strong antimicrobial properties against both the gram-positive and gram-negative uropathogenic bacterias, *Staphylococcus saprophyticus* and *Escherichia coli*. While less potent than the commercial antibiotic Nitrofurantoin, squid ink Alkaloids still show great potential as antimicrobial agents.

4. CONCLUSIONS AND RECOMMENDATIONS

The result of the experiment showed that there was a significant difference in the Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC) effect between each of the concentrations of *Uroteuthis edulis* crude extract and positive control (nitrofurantoin) for both *Staphylococcus saprophyticus* & Escherichia coli. The disk with the highest concentration (1000 µg/mL) of *Uroteuthis edulis* (Squid) ink extract exhibited the most potent inhibitory effect against both bacteria. In conclusion, using marine-derived compound alkaloid from *Uroteuthis edulis* was proven to have in vitro bactericidal effects on uropathogenic bacteria.

Additionally, the research underscores the importance of further research and testing, highlighting the potential for squid ink compounds to emerge as a significant antimicrobial agent. It introduces a novel strategy using natural compounds to address antibiotic resistance in treating urinary tract infections. The researchers strongly recommend isolating other compounds from *Uroteuthis edulis* to identify additional phytochemical constituents that could inhibit uropathogenic bacteria. It is also vital to evaluate the efficacy of the squid ink crude extract against other uropathogenic bacteria such as *Klebsiella pneumoniae*, *Proteus mirabilis*, and *Enterococcus faecalis*. The researchers also suggest considering geographic and seasonal variations and external environmental factors affecting the antimicrobial properties of *Uroteuthis edulis*. It is important to note that this study focused Further research would be needed only on the in vitro MIC and MBC of the extract in vivo.

5. CONFLICT OF INTEREST

There are no conflicts of interest.

6. ETHICAL APPROVAL

All authors hereby declare that "Principles of laboratory animal care" (NIH publication No. 85-23, revised 1985) and specific national laws, where applicable, were followed. All experiments have been examined and approved by the appropriate ethics committee**.**

7. ACKNOWLEDGMENTS

First and foremost, indebted to God, the Almighty, for His unceasing guidance and blessings, which enabled the investigation to be completed.

Their research adviser, Mr. Daniel Bercede, contributed invaluable expertise and confidence. They also appreciate the help and resources provided by the parents who unconditionally supported them throughout their studies. Hejlsberg Junn Razal, their statistician, provides accurate and reliable data. They are grateful to Far Eastern University and its laboratory for sharing knowledge and making materials easily accessible to them. Researchers' mutual appreciation, which strongly emphasised teamwork, communication, understanding, and listening, helped them advance despite obstacles.

8. DOCUMENTATION

Figure 1.
Dissection of *Uroteuthis edulis* squid.

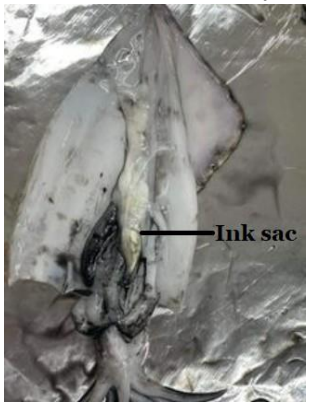

Figure 2. Extraction of *Uroteuthis edulis* squid ink.

Figure 3.
Lyophilisation of *Uroteuthis edulis* squid ink.

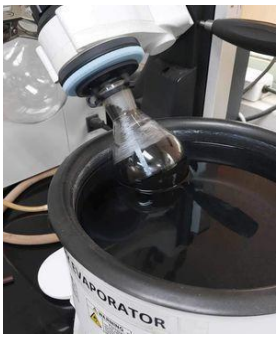

Figure 4.
Microplate of the MIC containing the positive control, negative control and varying concentration of the squid ink; 1000µg/mL, 500µg/mL, 250µg/mL, 125µg/mL, 62.5µg/mL, 31.25µg/mL, 15.63µg/mL, 7.81µg/mL, 3.91µg/mL, and 1.95µg/mL.

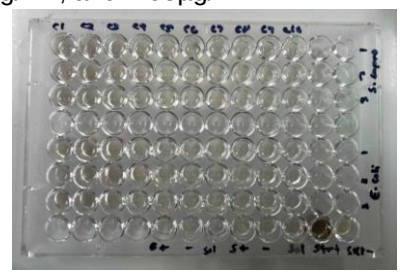

Figure 5.
The positive control exemplifies the minimum bactericidal concentration, which is Nitrofurantoin and squid ink concentrations.

(a.) *Staphylococcus saprophyticus*

(b.) Escherichia coli

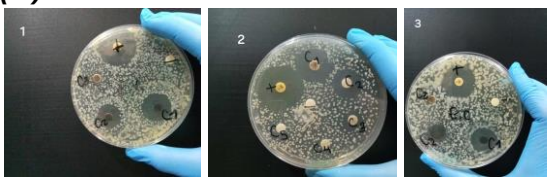

Figure 6.
Uroteuthis edulis squid ink is the final product after a series of tests.

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