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STANDARDIZATION OF SECONDARY METABOLITES AND HEAVY METAL CONTAMINATION ASSAY ON ONCHIDIID SLUG (*ONCHIDIUM TYPHAE*) IN WEST KALIMANTAN WATERS

Bambang Wijianto[1], **Annisa Larasati Nurhidayah**[1], **Sri Luliana**[2]

[1]Pharmacy Chemistry Department, Pharmacy Study Program, Universitas Tanjungpura, Pontianak, Indonesia
[2]Pharmacy Biology Department, Pharmacy Study Program, Universitas Tanjungpura, Pontianak, Indonesia

bam.wijianto@gmail.com

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ABSTRACT
Onchidiid slug or *Onchidium typhae* is a marine animal commonly found in West Kalimantan waters. This gastropod is commonly used as medicine and cosmetic by the locals. This research aims to assure the content and quality of *O. typhae* methanol extract by determining its secondary metabolites and heavy metal contamination. Phytochemical screening of alkaloid, steroid/ triterpenoid, flavonoid, tannin, and saponin are done using reagent tests. Chromatography profile was completed using silica gel G_{60} F_{254} plate and hexane: ethyl acetate: methanol as mobile phase. Heavy metal contamination analysis was performed using atomic absorption spectrophotometry to determine Hg, As, Cd, and Pb content. The phytochemical screening and chromatography profile of *O. typhae* methanol extract confirm the presence of alkaloids and steroids. Heavy metal analysis stated Hg, As, Cd, and Pb content in *O. typhae* are < 0.0008; < 0.001; < 0.001; and 0.05 ppm respectively. Since heavy metal levels are below the maximum contamination limit, it qualifies as a natural product ingredient.

Keywords: Heavy Metal Contamination Assay; Standardization of Secondary Metabolites; Onchidiid slug; *Onchidium typhae*

1. INTRODUCTION

The Onchidiid slug of the species *typhae* (*O. typhae*) is a gastropod of the family Onchidiidae. These marine animals are found in the seas of West Kalimantan and are exported as raw materials for medicines and cosmetics (Fernández et al., 1996; Guan et al., 2013; Wijianto et al., 2022). Thus, *O. typhae* in the waters of West Kalimantan has the potential to become a commodity with high economic value. In previous publications, the potential of onchidiid slug was known as antibacterial. Bacterial growth inhibitory activity is known to be sensitive to gram-positive and gram-negative, represented by *S. aureus* and *E. coli*, respectively. In addition, it was also known that the extracts of methanol, ethyl acetate, and chloroform had antifungal activity against *C. albicans*. Alkaloids, steroids, and flavonoids are thought to be responsible for antibacterial and antifungal activity (Wang et al., 2021; Wijianto et al., 2022; Zhou et al., 2018).

Water pollution, especially in the oceans with heavy metals, is one of the critical environmental issues. Heavy metals are essential elements that make up many biochemical structures and functions. The heavy metals include zinc (Zn), copper (Cu), nickel (Ni), molybdenum (Mo), manganese (Mn), chromium (Cr), and iron (Fe) (Meharg, 2011). On the other hand, non-essential metals such as lead (Pb), cadmium (Cd), mercury (Hg), and arsenic (As) have no known biological function and are even toxic to living things at low concentrations (C. Li et al., 2019; Shahid et al., 2015; Yi et al., 2011). However, recent research shows that there is a dramatic increase in cases of heavy metal pollution in the ocean due to anthropogenic activities

(Naser, 2013; Sankhla et al., 2016; Tchounwou et al., 2012). Illegal mining activities, factory waste disposal, and chemical fertilizers containing heavy metals are the most significant contributors to environmental pollution (Q. Li et al., 2010; Shahid et al., 2017). Marine products that have pharmacological potential are contaminated with heavy metals due to water pollution. In processed products, heavy metal contamination might start with the drying, storage, shipping, and maintenance procedures (Lajayer et al., 2017).

With its pharmacological potential and high economic value, it is necessary to standardize the raw material for onchidiid slug. The standardization is devoted to the content of heavy metal contamination, which has never been done before on the ethanolic extract of onchidiid slug from West Kalimantan waters. The results of this study are expected to be the basis for the development of derivative products from onchidiid slug, as well as raw materials for onchidiid slug for export activities.

2. METHODS

2.1. Material

The main material used in this research was fresh *O. typhae* slugs from Sambas districts. Methanol (p.a) Merck was used to extract the process. Mayer, Wagner, Dragendorff, Lieberman-Burchard, $FeCl_3$ 5% (Merck), acetic acid (Merck), sulfuric acid (Merck), chloroform (Merck), ammonia (Merck), Magnesium (Merck), and chloric acid (Merck) were used as reagents. Chromatography profile was done using silica gel G_{60} F_{254} plate (Merck) with hexane (Merck), ethyl acetate (Merck), and methanol (Merck) as the mobile phase. All reagent materials are original Merck products from Germany.

Heavy metal contamination testing was carried out based on SNI 6989.78:2019, SNI 06-6989.54-2005, and SNI 6989.84:2019 using an atomic absorption spectrophotometer. Other supporting instruments in this study consisted of a micropipette (Smart), oven (Memmert), grinder (Nankai), hotplate (Schott), rotary evaporator (Buchi), Buchner pump (Rocker Chemker 800), chamber (CAMAG), UV lamp (CAMAG), analytical balance (Ohaus), and the glassware.

2.2. Sample Preparation

Fresh *O. typhae* slugs were washed with water and cleaned in the innards. The prepared flesh was then put in hot water while stirring occasionally to get rid of the mucus. The flesh is then drained and dried before putting it in the 60 ℃ ovens for a day. Dried *O. typhae* was then grinded into fine powder.

2.3. Extraction

The sample extraction process used the maceration method. Extraction was done by submerging 160 grams of *O. typhae* powder with 500 mL methanol. The solvent was filtered and replaced with methanol for 3 x 24 hours. The collected filtrate was then reduced by a rotary evaporator and a water bath to produce a viscous extract.

2.4. Verification of Analysis Method

2.4.1. Linearity Test

The concentration of the standard solution was made at 12 ppm; 15 ppm; 18 ppm; 21 ppm; 24 ppm; and 27 ppm. The standard solution was measured using AAS at the wavelength of each metal being measured, namely Pb, Hg, Cs, and Cd. Measurements were taken 3 times. A standard curve that expresses concentration (x) vs absorbance (y) is constructed. To determine the value of the correlation coefficient, it employs the linear regression equation **y=bx+a**. A good linearity value is indicated by a correlation value that is close to or equal to 1. according to AOAC (2005), the criteria for acceptance of the linearity test are $r > 0.9900$ (AOAC International, 2011; Gamal, 2020; Harmono, 2020; Karnakar et al., 2020).

2.4.2. Test Accuracy

The data from the standard curve measurements that have been carried out can be used to calculate the percentage of the recovery. According to ICH, the accepted accuracy value is 98-102% (Gamal, 2020; Harmono, 2020; Karnakar et al., 2020).

$$\% \, Recovery = \frac{analysis \; result}{true \; value} x \; 100\%$$

2.4.3. Precision Test

The precision test is done by measuring the standard solution of various concentrations as in the linearity test with 3 replications and then it was calculated. Precision is a parameter to determine whether the instrument's response to the analyte is repeatable. According to ICH, the acceptable % RSD value is < 2% (AOAC International, 2011; Gamal, 2020; Harmono, 2020; Karnakar et al., 2020).

$$\% \, RSD = \frac{SD}{mean} x \; 100\%$$

2.4.4. Limit of Quantification (LOQ) and Limit on Detection (LOD)

LOD is the lowest concentration of analyte that can be detected, expressed in concentration units. LOQ is the lowest analyte concentration that can be accurately quantified, expressed in units of concentration. LOQ is directly related to accuracy and precision (AOAC International, 2011; Gamal, 2020; Harmono, 2020; Karnakar et al., 2020).

$$LOD = \frac{3xSD}{b}$$

$$LOQ = \frac{10xSD}{b}$$

SD = standard deviation
b = slope

2.5. Phytochemical Screening

2.5.1. Alkaloid Test

The extract was dissolved in chloroform in a ratio 1:10. Ammonia-chloroform and 2 drops of chloric acid 2N were added. The mixture was shaken thoroughly before collecting the chloroform layer. The liquid was distributed evenly to three test tubes. Meyer, Dragendorff, and Wagner reagents were then added to each solution. White, orange, and white color indicate positive results, respectively (Harborne, 1987).

2.5.2. Steroid/Triterpenoid Test

The extract was dissolved in methanol in a ratio 1:10 and followed by 1 mL of acetic acid until two layers formed. A drop of sulfuric acid was added carefully from the inside wall of the test tube until a change of color appeared before shaking the mixture. Green-blue color indicates the presence of steroid, while purple-red indicates triterpenoid (Harborne, 1987).

2.5.3. Flavonoid Test

Methanol is added to extract until dissolved in a ratio 10:1. Mg band is added to the solution before chloric acid and let the mixture react. Red-yellow color implies a positive reaction for flavonoid (Harborne, 1987).

2.5.4. Tannin Test

The extract is dissolved with methanol in a ratio 1:10 and then 5 drops of FeCl3 5% were added. Blackish green color indicates a positive reaction for tannin (Harborne, 1987).

2.5.5. Saponin Test

Methanol was used to dissolve extract in a ratio 10:1 and then distributed to a test tube. 5 mL of hot water was added to the extract and then shaken thoroughly for a minute. Formed froth was

observed for 10 minutes and after the addition of 2 drops of chloric acid 2N. A positive reaction is a stable froth/foam (Harborne, 1987).

2.5.6. Chromatography Profile

Silica gel G_{60} F_{254} plate was used as the stationary phase. Chamber is filled with 20 mL of hexane, ethyl acetate, and methanol mixture (1:2:2) as the mobile phase. The eluted plate was observed under 254 nm and 366 nm UV lights. Stain reagents used were Dragendorff for alkaloid, $AlCl_3$ 5% for flavonoid, $FeCl_3$ 5% for tannin, and Liebermann-Bouchard for steroid (Wagner & Bladt, 1996).

2.5.7. Heavy Metal Contamination Assay

A qualitative test was done by adding KI 10%, NaOH, and HCl separately to the diluted extract (Harmawan & Lestari, 2020; Prayoga et al., 2021; Svehla, 1990). Qualitative analysis was done using atomic absorption spectrophotometry (AAS). 1 gram of *O. typhae* powder was destructed with 10 mL of HNO_3, 6 mL of sulfuric acid, and 1 mL of hydrogen peroxide. Particularly to test Hg metal, the sample used additional $KMnO_4$ and $SnCl_2$. 5 mL of concentrated H_2SO_4 and 2.5 mL of concentrated HNO_3 were put into each Erlenmeyer, then 15 mL of $KMnO_4$ solution was added and wait for 15 minutes. The mixture was then heated with a hotplate until white smoke appeared. The destructed sample was added to a 500 mL volumetric flask and filled with aquadest until the measured line.

Series of concentration solutions are made from 1000 ppm standard solution. 10 mL of solution was added with diluted HNO_3 to obtain 100 ppm solution. This solution was diluted further to make 0.01; 0.05; 0.1; 0.15; 0.3; and 0.5 ppm solution. Absorbance was measured with AAS to form a calibration curve. Solution was diluted or concentrated if needed (Badan Standardisasi Nasional, 2005, 2019a, 2019b).

3. RESULTS AND DISCUSSION

The extraction of *O. typhae* powder using methanol yields 9.05% of viscous extract. This percentage doesn't stray far from previous research of 10%. The high-water content of onchiidid slug meat causes low yields after being dried and mashed. The results of the organoleptic test of *O. typhae* extract are shown in **Table 1**.

Table 1. Organoleptic test result on *O. typhae* extract

Parameters	Description
Scent	Distinctive aromatic
Taste	Tasteless
Color	Yellow-ish dark green
Form	Viscous

Phytochemical screening was done to determine secondary metabolites in methanol extract of *O. typhae*. Compounds that were tested include alkaloid, steroid/triterpenoid, flavonoid, tannin, and saponin. The result is *O. typhae* methanol extract contains alkaloid and steroid. Results are shown in **Table 2.**

Table 2. Results of phytochemical screening of *O. typhae* methanol extract

Compound(s)	Reagents	Result
Alkaloid	Meyer	+
	Dragendorff	+
	Wagner	+
Steroid	Mg + HCl	+
Triterpenoid		-
Flavonoids	$CH_3COOH + H_2SO_4$	-
Tannin	$FeCl_3$ 5%	-
Saponin	Hot water and HCl 2N	-

Alkaloid in gastropod has been documented in Jorunna funebris where joumycin, an isoquinoline alkaloid, has been successively isolated. The results of this study are in line with previous studies that The *Onchidium* genus (Mollusca, Gastropod, Pulmonata, Systellommatophora, Onchidiidae family) is reported to have 60 active compounds including polypropionates, depsipeptides, terpenoids and other chemical components. Many biological activities of Onchidium such as anticancer, anti-viral and anti-bacterial and anti-fungi activities have been reported (Fernández et al., 1996; Fontana et al., 2000; Wang et al., 2021; Wijianto et al., 2022; Zhou et al., 2018). The same slug of *O. typhae* has been reported to contain alkaloid using tube tests (Wijianto et al., 2022).

In the Asia pacific coastline, *O. typhae* is an economically important slug in the local area due to its high nutritional and medicinal value. They are considered a high-grade food due to the characteristics of high protein, low fat, aphrodisiac, and digestive functions. With the economic value possessed, many people depend on this commodity for their economic life for domestic and export needs.

The chromatography profile was completed by separating compounds in extract according to their polarity. Mobile phase used in this study was hexane-ethyl acetate-methanol with the ratio of 1:2:2. This combination can separate spots on the plate. Results are shown in **Figure 1**.

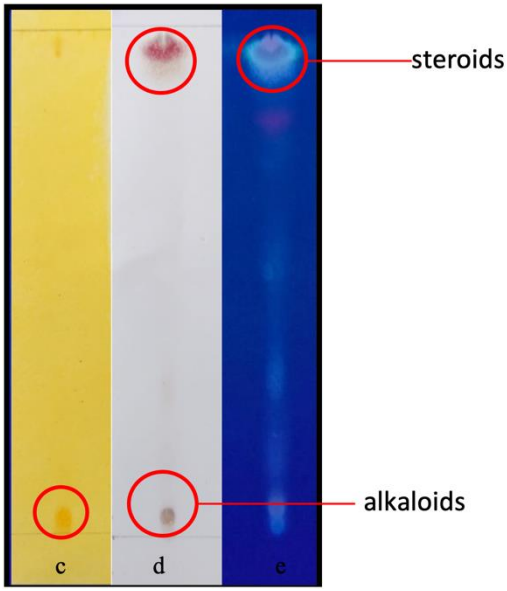

Figure 1. Profile of onchidiid slug methanol extract; c) sprayed with Dragendorff; d) sprayed with Liebermann Buchard; e) after sprayed with LB

The stain reagent result showed an orange spot with Dragendorff and purple spot with Liebermann-Bourchard after heating. This confirms previous research and tube testing that demonstrate secondary metabolites in *O. typhae* methanol extract are alkaloid and steroid.

Heavy metal in the sample was tested both qualitatively and quantitatively. A qualitative test showed that sample was positive with Pb and Hg from the formation of yellow precipitation with NaOH and KI reagents (Harmawan & Lestari, 2020; Prayoga et al., 2021). Quantitative analysis was carried out with AAS method. The results show that Hg, As, and Cd metal levels are low and below the limit of detection. Pb metal was detected to be 0.05 ppm in the sample. The level of heavy metal contamination in *O. typhae* sample is still below the maximum limit. This is probably due to the sample's low concentration of metal contaminants. Results of heavy metal content can be seen in **Table 3**. Many publications on heavy metal contamination show positive results for heavy metal content with concentrations that are harmful to health. Due to pollution from industrial waste, unfavorable natural conditions are the primary source of the general heavy metal concentration in marine products.

Table 3. Results of heavy metal level in *O. Typhae*

Metals	Results (ppm)	Max. limit (ppm)
Hg	< 0.0008	≤ 0.50
As	< 0.001	≤ 5
Cd	< 0.001	≤ 0.30
Pb	0.05	≤ 10

4. CONCLUSION

O. typhae methanol extract contains alkaloid and steroid compounds and has Hg, As, Cd, and Pb contents below the maximum limit. Recent studies showed that exposure to mixed metals such as metalloid/arsenic alloys, lead, and cadmium produces severe effects at both relatively high and low doses using biomarker-specific analytical methods. The effects are influenced by dose, duration of exposure, and genetic factors. Another study also revealed that co-exposure to cadmium and inorganic arsenic resulted in more severe kidney damage than exposure to either element alone (C. Li et al., 2019). Therefore, further research is needed using specific biomarker analysis methods as well as additional investigation to clarify the molecular mechanism and effects of human exposure to hazardous metal compounds on public health.

5. ACKNOWLEDGMENT

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6. CONFLICT OF INTEREST

The author declares that there are no competing conflicts of interest.

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