Influence of Extraction Solvents on Capsaicin Compound and Cytotoxic Activity of *Piper retrofractum Vahl*

Kirana Anggraini[1] (✉), Dyah Ayu Woro Setyaningrum[1], Laela Wulansari[1,2], Hening Tyas Andayani[3], Laviany Putri Shihran[1], and Isra Fauziyyah[1]

[1]Universitas Trisakti, Jakarta, Indonesia
kirana_anggraeni@trisakti.ac.id

[2]Tropical Biopharmaca Research Center, Institute for Research and Community Service (LPPM) IPB University, Bogor, Indonesia
[3]Fachärztin der Innere Medizin, Städtische Kliniken, Mönchengladbach, Germany

Abstract. Capsaicin (8-Methyl-N-vanillyl-trans-6-nonenamide) is an active compound found in plants from the genus Capsicum. Genus capsium posseses different species of red chilli (*Capsicum annuum Linn*), green chilli (*Capsicum annuum var.annuum*) and other varieties that are familiar in Indonesia. However, the plants were not originally from Indonesia but from American Continent. Indonesian ancestor utilized chilli to add spiciness on foods which refers to Piper retrofractumVahl or Javanese chilli. Capsaicin has positive effects on health such as analgesic, cardioprotective, anorexigenic, chemopreventive, chemotherapy, and cardioprotective effects. In addition, capsaicin has beneficial effects in maintaining glucose levels, insulin homeostasis, reducing itching and symptoms in Non-Allergic Rhinitis (NAR), and an alternative therapy for neurogenic bladder. This research is aimed to acknowledge if capsaicin is also discovered in Javanese chilli. The method used to obtain the extract was by maceration of Java chilies using 30%, 70%, and 96% ethanol, n-hexane, ethyl acetate, and water as solvents. The research was started by conducting phytochemical and moisture content tests on simplicia. Then, each extract was tested for cytotoxic activity on shrimp larvae and the determination of capsaicin levels by High Performance Liquid Chromatography (HPLC). The results showed that the highest yield of Java chili extract was 51.14% in ethanol 70%. Phytochemical testing of Java chili simplicia contains flavonoids and saponins. The ethanol 70% extract had the highest cytotoxic activity with an LC50 value of 205.21 ppm. The highest concentration of capsaicin was found in ethyl acetate solvent of 785.58 mg/g followed by ethanol 70% at 486.06mg/g.

Keywords: Extraction Solvents, Capsaicin Compound, Cytotoxic Activity, Piper retrofractum Vahl

1 Introduction

Capsaicin (8-Methyl-N-vanillyl-trans-6-nonenamide) is the active compound found in hot peppers that gives chilies their hot taste [1]. Capsaicin was first purified in 1876 but

M. Setiyo et al. (eds.), *Proceedings of the 4th Borobudur International Symposium on Science and Technology 2022 (BIS-STE 2022)*, Advances in Engineering Research 225,
https://doi.org/10.2991/978-94-6463-284-2_89

its structure began to be described in 1919 [2]. Due to its chemical structure, capsaicin is well absorbed up to 94% when administered topically or orally [3]. The use of spice plants is also influenced by the level of knowledge, age, education level, economic status, environmental factors, and sources of information/information media [4]. Studies demonstrate the efficacy of capsaicin as an analgesic. Treatment with capsaicin is effective in various types of pain conditions such as complex regional pain syndromes, neuropathic pain postsurgical neuropathic pain [5] post-herpetic neuralgia [6] and painful diabetic peripheral neuropathy [7]. Research has also shown capsaicin to be effective in weight loss and obesity improvement [8]. Capsaicin also has beneficial effects on glucose levels, insulin homeostasis, and diabetes [9].

The evidence showed the beneficial effects of capsaicin on the cardiovascular system [10]. Capsaicin activates TRPV1 which can stimulate the release of CGRP, which is the most powerful vasodilator that regulates blood pressure in both physiological and pathophysiological conditions, producing a blood pressure-lowering effect [11]. Capsaicin has been shown to inhibit platelet aggregation [12], which may also provide protection against cardiovascular disease [13]. Long-term activation of TRPV1 by capsaicin will inhibit the formation of foam cells so that it will reduce lipid storage and atherosclerotic lesions in the aortic sinus and thoracoabdominal aorta of rats, and ultimately slow down the process of atherosclerosis. The antioxidant properties of capsaicin contribute to its protective effect on the cardiovascular system. LDL oxidation is an early factor for the formation and development of atherosclerosis [14].

Capsaicin has been shown to have chemopreventive and chemotherapeutic effects [15]. Capsaicin also works on itching with nostalgia paresthetica [16], and neuropathic pruritus [17]. Capsaicin has a gastroprotective effect by modulating sensory neurons [18]. Capsaicin has been studied as an alternative therapy for symptomatic relief of neurogenic bladder, a urological disorder that seriously affects patients' quality of life [19]. The clinical use of capsaicin is also successful in patients with detrusor bladder hyperreflexia with multiple sclerosis, as well as after spinal cord injury [20].

Capsaicin is discovered in various type of capsium genus plants such as red chilli (Capsicum annuum Linn), green chilli (Capsicum annuum var. annuum) and other varieties known in Indonesia. Capsicum is not originally from Nusantara, instead it comes from American Continent. Indonesian ancestors used chilli to add spiciness on food currently refers to Piper retrofractum Vahl or green chilli. This research was conducted to determine the capsaicin content in Javanese chilies, as well as to find out the best solvent in the extraction process from Javanese chilies which produced the highest capsaicin levels.

2 Method

2.1 Place, Collecting, and Determining Samples

The research material was the fruit of the Javanese chili plant taken from the Biopharmaca Cultivation Conservation Unit (BCCU) of the Tropical Biopharmaca Research Center, LPPM IPB, which was obtained from Java which was harvested at the age of

3-4 months after flowering. The determination was carried out at the Biopharmaca Cultivation Conservation Unit (BCCU).

2.2 Simplicia Making

Three kilograms of fresh Java chilies were weighed, then washed and dried in direct sunlight for 4-5 days. After drying, the dry sorting is carried out, and mashed with a blender. The simplicia powder obtained was sieved using an 80-mesh sieve and then weighed. The simplicia powder is then stored in a clean, dry container and protected from sunlight for the next extraction process.

2.3 Production of Java Chilli Extract

Extract preparation and testing were carried out at the Laboratory of the Tropical Biopharmaca Research Center, Institute for Research and Community Service (LPPM), IPB University. Samples of Java chili simplicia were weighed for extraction with 10 grams of various solvents each, then added solvents namely 96%, 70%, 30% ethanol, 500 mL of water, ethyl acetate, and n-hexane. Maceration was carried out 2 x 24 hours with several times of stirring, then it was filtered. The collected filtrate was concentrated using a vacuum rotary evaporator at 45-50˚C to obtain a viscous ethanol extract of 96%, 70%, 30%, water, ethyl acetate, and n-hexane extract.

2.4 Water content

Two grams of Javanese chili simplicia were weighed in a container with a constant weight. Then, it was heated in an oven at + 105 degrees Celsius for 3 hours. After that, it was cooled in a desiccator and weighed until it reached a constant weight.

2.5 Yield Analysis of Javanese Chilli Extract

The yield of Javanese chili extract was calculated by comparing the weight of the Javanese chili extract with the weight of the Javanese chili simplicia used for extraction.

2.6 Phytochemical Screening

Phytochemical screenings were carried out for alkaloids, flavonoids, tannins, saponins, triterpenoids, and steroids based on the method of Harborne 1987.

2.7 Alkaloids

A total of 0.5 grams of condensed extract or 1 gram of simplicial was dripped with 3-5 drops of ammonia, then 5 mL of chloroform was added, then it was homogenized and filtered. The filtrate obtained was added with 2M Sulfuric acid reagent, then homogenized. The top layer was taken and used as an experimental solvent which was then

treated as follows: 1) Experimental solvent 1 was added with 2 drops of Mayer's reagent, a positive result indicated by the formation of a white precipitate. 2) Experiment solvent 2 was added with 2 drops of Dragendorf reagent, and a positive result was indicated by the formation of an orange precipitate. 3) Experiment solvent 3 wasadded with 2 drops of Wagner reagent, and a positive result was indicated by the formation of a brown precipitate.

2.8 Flavonoids, Tannins, and Saponins

0.5 grams of condensed extract or 5 grams of simplicial was dissolved in distilled water and heated for 5 minutes. Then the solvent was filtered and the filtrate was divided into 3 parts. To test the flavonoid filtrate, Mg powder and HCl : Ethanol (1:1) were added, followed by amyl alcohol. The color formed on the amyl alcohol layer was observed, if a yellow, orange or red coloris formed then it consists of flavonoids. For the tannin filtrate test, 3 drops of 10% FeCl3 were added, if a greenish-black color is formed then it consists of tannins. For the saponin test, the filtrate was shaken for 10 seconds. A positive result is indicated by the formation of stable foam for over 2 minutes.

2.9 Triterpenoids and Steroids

0.5 grams sample of condensed extract or 1 gram of simplicial was dissolved in ethanol and heated for 5 minutes, then the sample was filtered into a porcelain dish. The filtrate was heated to dryness and then1 mL of diethyl ether, 1 drop of acetic anhydrous, and 1 drop of concentrated sulfuric acid were added. A positive reaction is indicated by the formation of a red/purple solvent for triterpenoids and blue or greenforsteroids.

2.10 Quinone

0.5 grams of condensed extract or 1 gram of simplicial was added to methanol then it was heated and filtered. The filtrate results were added with 3 drops of 10% NaOH. A positive reaction is indicated by the formation of a red color for hydroquinone [21].

2.11 Cytotoxic Activity Test on Shrimp Larvae

The cytotoxic activity test by determining the LC50 value was carried out using Artemia salina shrimp eggs. A. salina used for the toxicity test was obtained from hatching using seawater with the help of an aerator to meet dissolved oxygen levels. The extract toxicity test was carried out using A. salina shrimp larvae. The shrimp larvae used were aged 48 hours after the shrimp larvae hatched. A. salina cysts of as much as ± 50 mg were put into a container containing seawater that had been filtered and equipped with an aerator. The cysts were left for 48 hours under light to hatch completely. After hatching, 10 A. salina larvae were put into a 2 ml vial, then it was added a stock extract solution with a concentration of 4000 ppm and adjusted the volume with seawater so that the final concentration of the extract was 0, 10, 100, and 1000 ppm. After 24 hours,

the number of dead larvae was counted. The lethal concentration (LC) value was determined by the probit analysis method with a 95% confidence interval [22].

2.12 Determination of Capsaicin content with High Performance Liquid Chromatography (HPLC)

Standard and sample preparation. Standard capsaicin was dissolved in methanol to make a concentration of 100 ppm. Each extract was weighed as much as 0.1 gram and then 8 mL of methanol solvent was added and sonicated for 1 hour. Then the sample solution was filtered into a 10 mL flask and calibrated with methanol up to 10 mL. After that, it was filtered with 0.45 micrometer Whatman filter paper and injected into HPLC as much as 20 μL.

Identification by HPLC. The mobile phase used was methanol and aquabides with a composition ratio of 80:20. The wavelength was 235 nm, while the flow of the mobile phase was 1 mL/minute.

3 Result and Discussion

3.1 Plant Determination

The test sample was identified at the Biopharmaca Cultivation and Conservation Unit (BCCU) of the Tropical Biopharmaca Research Center, Institute for Research and Community Service (LPPM) IPB University, it showed that a sample of Piper retrofractum Vahl was from the Piperaceae tribe.

3.2 Phytochemical compounds

Tests for the content of phytochemical compounds were carried out on simplicia, which contained saponin and flavonoid. The positive reactions in the flavonoids, saponins, and tannins indicate the presence of phenol groups

3.3 Water Content and Extraction

The water content results of the Javanese chili simplicia were 8.75%, fulfilling the quality requirements and can be used for further analysis. Removing the water content up to a certain amount is useful for extending the durability of simplicia. Extremely high water content can become a medium for the growth of microorganisms that cause damage to the simplicia [23].

3.4 Rhizome Content on Different Types of Extract

The yield of Javanese chili extract is presented in Table 1, where the highest yield was found in 70% ethanol extract.

Table 1. The yield of Javanese chili extract

	ETOH 30%	ETOH 70%	ETOH 96%	Water	Ethyl acetate	N hexane
Extract(g)	7.59	51.14	8.44	11.85	6.33	8.30

3.5 Shrimp Larvae Cytotoxic Activity Test on Different Types of Extracting Solvent

Shrimp larvae toxicity tests were carried out to observe the potential bioactivity and cytotoxic activity of each extract so that a safe extract concentration could be determined for further testing. In addition, extracts that exhibit toxic properties can be developed as anti-cancer drugs [24]. A plant extract will be bioactive and have anti-cancer potential if it has an LC_{50} value of less than 1000 ppm. Based on Table 2, it can be seen that all Javanese chili extracts have the potential as bioactive compounds and can be used as medicine. It is due to the fact that each extract produced an LC_{50} of less than 1000 ppm so that, at low concentrations, it was able to kill 50% of the population of A. salina shrimp larvae. The highest bioactive potential and the toxic extract was 70% ethanol extract because it had the lowest LC_{50} value, which was 205.21 ppm, which means that, at a small concentration, this extract can kill half the population of A. salina shrimp larvae. The LC_{50} value is the highest concentration limit for determining various extract concentrations in subsequent tests [21].

Table 2. LC50 value of Javanese chili extract on A. Salina larvae

	ETOH 30%	ETOH 70%	ETOH 96%	Water	Ethyl acetate	Water
LC_{50} (ppm)	434.57	205.21	334.32	650.186	443.03	292.78

3.6 Capsaicin Content on Different Types of Extract

The results of the calculationof the capsaicin compound concentration in each extract can be seen in Fig 1. The highest capsaicin content was found in ethyl acetate solvent of 785.53mg/g followed by 70% ethanol of 426.34mg/g and the lowest in water solvent of 6.55mg/g. It is due to the fact that capsaicin is a non-polar phenolic compound so it cannot dissolve in water. The main solvents used to extract and maintain the properties of capsaicin are nonpolar solvents such as ether, benzene, dimethyl sulfoxide, and acetone, as well as ethanol due to their mixed nature [3].

The content of capsaicin in each extract of Javanese chilies in this study was higher than in previous studies conducted in Nepal and Saudi Arabia. In Nepal, research conducted on 16 types of chilies (Capsium fruit) found capsaicin levels ranging from 2.19 to 19.73 mg/g [25]. While research conducted in Saudi Arabia on hot chili, red chili, green chili, green pepper, red pepper, and yellow pepper found capsaicin levels range from 0.001 to 4.24 mg/g [26]. The difference related to the content of capsaicin

in this study and the previous study could be due to differences in plant ecological conditions, varieties, harvesting ages, simplicia manufacturing methods, and extraction methods used.

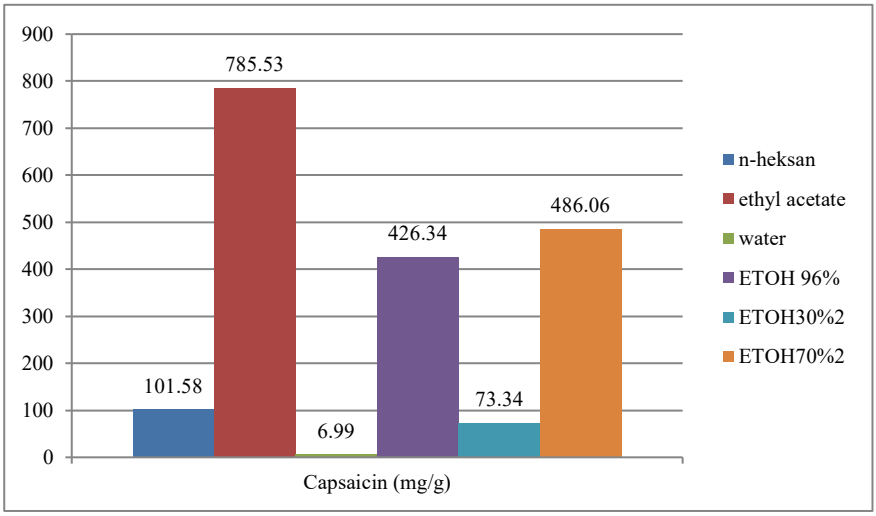

Fig. 1. Graph of capsaicin levels of various Javanese chili extracts

4 Conclusion

Capsaicin is discovered on Piper retrofractum Vahl fruits. The secondary metabolites in Javanese chili simplicia are flavonoids and saponins. The highest yield of extract was 11.85 g in a water solvent. The highest bioactive potential and cytotoxic activity extract is a 70% ethanol extract with an LC50 of 205.21 ppm. The highest content of capsaicin was found in ethyl acetate solvent of 785.53mg/g followed by ethanol 70% at 486.06mg/g.

Acknowledgment. We thank Trisakti University for funding this research through the Faculty Research grant (PUF) Number: 322/A.1/LPT/USAKTI/II/2022 on behalf of dr. Kirana Anggraini MKM

References

1. E. K. Nelson and L. E. Dawson, “The constitution of capsaicin, the pungent principle of capsicum (III),” *J. Am. Chem. Soc.*, vol. 45, no. 9, pp. 2179–2181, Sep. 1923, doi: 10.1021/ja01662a023.
2. E. K. Nelson, “The constitution of capsaicin, the pungent principle of capsicum,” *J. Am. Chem. Soc.*, vol. 41, no. 7, pp. 1115–1121, Jul. 1919, doi: 10.1021/ja02228a011.

3. P. Suresh and K. Srinivasan, "Tissue distribution & elimination of capsaicin, piperine & curcumin following oral intake in rats," *Indian J. Med. Res.*, vol. 131, pp. 682–691, May 2010.
4. M. Adiyasa and M. Meiyanti, "Pemanfaatan obat tradisional di Indonesia: Distribusi dan faktor demografis yang berpengaruh," *J. Biomedika dan Kesehat.*, vol. 4, no. 3, pp. 130–138, 2021, doi: 10.18051/JBiomedKes.2021.v4.130-138.
5. W. S. Kingery, "A critical review of controlled clinical trials for peripheral neuropathic pain and complex regional pain syndromes," *Pain*, vol. 73, no. 2, pp. 123–139, 1997, doi: https://doi.org/10.1016/S0304-3959(97)00049-3.
6. P. Zis, A. Apsokardos, C. Isaia, P. Sykioti, and A. Vadalouca, "Posttraumatic and postsurgical neuropathic pain responsive to treatment with capsaicin 8% topical patch," *Pain Physician*, vol. 17, no. 2, pp. E213–E218, 2014.
7. J. Kiani, F. Sajedi, S. Nasrollahi, and F. Esna-Ashari, "A randomized clinical trial of efficacy and safety of the topical clonidine and capsaicin in the treatment of painful diabetic neuropathy," *J. Res. Med. Sci. Off. J. Isfahan Univ. Med. Sci.*, vol. 20, no. 4, pp. 359–363, 2015.
8. Q. Yu *et al.*, "Expression of TRPV1 in rabbits and consuming hot pepper affects its body weight," *Mol. Biol. Rep.*, vol. 39, no. 7, pp. 7583–7589, 2012, doi: 10.1007/s11033-012-1592-1.
9. L.-J. Yuan *et al.*, "Capsaicin-containing chili improved postprandial hyperglycemia, hyperinsulinemia, and fasting lipid disorders in women with gestational diabetes mellitus and lowered the incidence of large-for-gestational-age newborns," *Clin. Nutr.*, vol. 35, no. 2, pp. 388–393, 2016, doi: https://doi.org/10.1016/j.clnu.2015.02.011.
10. N. Harada and K. Okajima, "Effects of Capsaicin and Isoflavone on Blood Pressure and Serum Levels of Insulin-Like Growth Factor-I in Normotensive and Hypertensive Volunteers with Alopecia," *Biosci. Biotechnol. Biochem.*, vol. 73, no. 6, pp. 1456–1459, Jun. 2009, doi: 10.1271/bbb.80883.
11. P.-Y. Deng and Y.-J. Li, "Calcitonin gene-related peptide and hypertension," *Peptides*, vol. 26, no. 9, pp. 1676–1685, 2005, doi: https://doi.org/10.1016/j.peptides.2005.02.002.
12. M. J. Adams, K. D. K. Ahuja, and D. P. Geraghty, "Effect of capsaicin and dihydrocapsaicin on in vitro blood coagulation and platelet aggregation," *Thromb. Res.*, vol. 124, no. 6, pp. 721–723, Dec. 2009, doi: 10.1016/j.thromres.2009.05.001.
13. D. L. Bhatt and E. J. Topol, "Scientific and therapeutic advances in antiplatelet therapy," *Nat. Rev. Drug Discov.*, vol. 2, no. 1, pp. 15–28, 2003, doi: 10.1038/nrd985.
14. R. Stocker and J. F. Keaney, "Role of Oxidative Modifications in Atherosclerosis," *Physiol. Rev.*, vol. 84, no. 4, pp. 1381–1478, Oct. 2004, doi: 10.1152/physrev.00047.2003.
15. R. Zhang, I. Humphreys, R. P. Sahu, Y. Shi, and S. K. Srivastava, "In vitro and in vivo induction of apoptosis by capsaicin in pancreatic cancer cells is mediated through ROS generation and mitochondrial death pathway," *Apoptosis*, vol. 13, no. 12, pp. 1465–1478, 2008, doi: 10.1007/s10495-008-0278-6.
16. H. H. Andersen, C. Sand, and J. Elberling, "Considerable Variability in the Efficacy of 8% Capsaicin Topical Patches in the Treatment of Chronic Pruritus in 3 Patients with Notalgia Paresthetica," *Ann Dermatol*, vol. 28, no. 1, pp. 86–89, Feb. 2016, [Online]. Available: https://doi.org/10.5021/ad.2016.28.1.86.
17. L. Misery *et al.*, "Successful treatment of refractory neuropathic pruritus with capsaicin 8% patch: a bicentric retrospective study with long-term follow-up," *Acta Derm. Venereol.*, vol. 95, no. 7, pp. 864–865, 2015, doi: 10.2340/00015555-2085.
18. P. Holzer and W. Sametz, "Gastric mucosal protection against ulcerogenic factors in the rat mediated by capsaicin-sensitive afferent neurons," *Gastroenterology*, vol. 91, no. 4, pp. 975–981, 1986, doi: https://doi.org/10.1016/0016-5085(86)90702-X.

19. H. Foster and A. Lake, "Use of Vanilloids in Urologic Disorders," *Prog. Drug Res.*, vol. 68, pp. 307–317, Jun. 2014, doi: 10.1007/978-3-0348-0828-6-13.
20. M. de Sèze, L. Wiart, P.-A. Joseph, J.-P. Dosque, J.-M. Mazaux, and M. Barat, "Capsaicin and neurogenic detrusor hyperreflexia: A double-blind placebo-controlled study in 20 patients with spinal cord lesions," *Neurourol. Urodyn.*, vol. 17, no. 5, pp. 513–523, Jan. 1998, doi: https://doi.org/10.1002/(SICI)1520-6777(1998)17:5<513::AID-NAU7>3.0.CO;2-G.
21. A. Harborne, *Phytochemical methods a guide to modern techniques of plant analysis*. Berlin, Heidelberg: Springer Dordrecht, 1998.
22. B. N. F. Meyer N R; Putnam, J E; Jacobsen, L B; Nichols, D E; McLaughlin, J L, "Brine Shrimp: A Convenient General Bioassay for Active Plant Constituents," *Planta Med*, vol. 45, no. 05, pp. 31–34, 1982, doi: 10.1055/s-2007-971236.
23. E. Yuslianti, B. Bachtiar, D. Suniarti, and A. Sutjiatmo, "Standardisasi farmasitikal bahan alam menuju fitofarmaka untuk pengembangan obat tradisional Indonesia," *Dentika Dent. J.*, vol. 19, no. 2, pp. 179–185, 2016.
24. J. L. Carballo, Z. L. Hernández-Inda, P. Pérez, and M. D. García-Grávalos, "A comparison between two brine shrimp assays to detect in vitrocytotoxicity in marine natural products," *BMC Biotechnol.*, vol. 2, no. 1, p. 17, 2002, doi: 10.1186/1472-6750-2-17.
25. B. Thapa, N. Skalko-Basnet, A. Takano, K. Masuda, and P. Basnet, "High-Performance Liquid Chromatography Analysis of Capsaicin Content in 16 Capsicum Fruits from Nepal," *J. Med. Food*, vol. 12, pp. 908–913, Sep. 2009, doi: 10.1089/jmf.2008.0187.
26. Z. Alothman, A. Badjah-Hadj-Ahmed, M. Habila, and A. Ghafar, "Determination of Capsaicin and Dihydrocapsaicin in Capsicum Fruit Samples using High Performance Liquid Chromatography," *Molecules*, vol. 16, pp. 8919–8929, Dec. 2011, doi: 10.3390/molecules16108919.