Brine Shrimp Lethality Assay of Ethyl Cinnamate Derivatives Synthesized by Microwave Irradiation of Cinnamic Acids with Ethyl Acetate

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Keywords: Microwave Irradiation, Ethyl Cinnamates, Brine Shrimp Lethality, Cinnamic Acids.

Abstract: The simple and low-cost bioassay of brine shrimp lethality assay can be used to examine the toxicity effect of potential drugs. Ethyl *p*-methoxycinnamate is a major compound that can be isolated from the rhizome of *Kaempferia galanga* which was suggested that its ethyl ester is the most important functional group contributing to its bioactivity. In general, the synthesis of this typical ethyl ester of cinnamic acids is derived from the reaction of ethanol and cinnamic acid under reflux with the contribution of acid as a catalyst. In this study, cinnamic acid and selected *para*-substituted cinnamic acid derivatives are subjected to microwave irradiation-guided ethyl esterification by using ethyl acetate and sulfuric acid. This alternative method for ethyl esterification yielded 19–39% products of ethyl cinnamate derivatives and ethyl *p*-chloro cinnamate showed the highest toxicity level among the tested ethyl cinnamate derivatives with LC_{50} of 1.29 µg/mL.

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1 INTRODUCTION

The simple and low-cost bioassay of brine shrimp lethality assay can be used to examine the toxicity effect of potential drugs (Michael, et al., 1956; Dash, et al., 2014). Ethyl *p*-methoxycinnamate is a major compound derived from *Kaempferia galanga* which is a main constituent as an anti-inflammatory agent (Elshamy, et al., 2019; Umar, et al. 2014). It is suggested by the study on the structure-activity relationship that the ethyl ester form of this cinnamic acid derivative is the most important functional group contributing to the anti-inflammatory activity (Komala, et al., 2018). In general, the synthesis of this typical ethyl ester of cinnamic acids is derived from the reaction of ethanol and cinnamic acid under reflux with the contribution of sulfuric acid (Anthony, et al., 2022). Ethyl acetate under sulfuric acid can be hydrolyzed (Jaques, 1971; Vinnik and Librovich, 1975) for presenting ethanol and can be donated as an ethyl ester group candidate for direct transesterification with cinnamic acid. Microwave irradiation is believed to increase the efficiency during organic synthesis by offering rapid temperature increments (Hoz, et al., 2005). To the best knowledge, no reported study has synthesized ethyl cinnamate by microwave irradiation of ethyl acetate and sulfuric acid. Since several cinnamic acid derivatives are relatively less soluble in the ethanol, thus by taking advantage of ethyl acetate hydrolysis under a high concentration of sulfuric acid this study then directly conducts the ethyl esterification of selected cinnamic acids with sulfuric acid as a catalyst under microwave irradiation. The representative ethyl ester of cinnamic acid derivatives

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Annur, R. M., Urribah, N., Meilawati, L., Hermawan, F., Salahuddin, S., Megawati, M. and Tachrim, Z. P.
Brine Shrimp Lethality Assay of Ethyl Cinnamate Derivatives Synthesized by Microwave Irradiation of Cinnamic Acids with Ethyl Acetate.
DOI: 10.5220/0013471800004612
Paper published under CC license (CC BY-NC-ND 4.0)
In *Proceedings of BRIN's 2nd International Conference for Health Research (ICHR 2024)*, pages 5-8
ISBN: 978-989-758-755-9
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then aims to understand its toxicity by utilizing a brine shrimp lethality assay.

2 MATERIALS AND METHODS

2.1 Generals

Hexane and ethyl acetate were distillate before use, the distilled ethyl acetate was further dried with $MgSO_4$ before reaction, conc. H_2SO_4 (96%, Merck), and reverse osmosis (RO) water were produced on-site. Non-modified domestic microwave oven 900-Watt (Moderna MG 2516) was utilized for the synthesis and the reaction mixture was covered with heat resistance glass before irradiation. Seawater was taken from Anyer, Banten, Indonesia, stored at room temperature, and filtered before use. NMR spectra (^{1}H and ^{13}C) were collected using AVANCE NEO 700MHz NMR (BRIN, Serpong, Indonesia). GC-MS data were collected using GC-MS Agilent 7890B GC and 5977A MSD (BRIN, Serpong, Indonesia).

2.2 Synthesis of Ethyl Cinnamate Derivatives via Microwave Irradiation of Cinnamic Acids with Ethyl Acetate

Cinnamic acid derivatives (**1–3**, 100 mg, 0.51–0.69 mmol) are dissolved in 20 mL ethyl acetate. 1 mL conc. H_2SO_4 is added to the cinnamic acid derivatives solution in the ice bath. This solution was then microwaves irradiated for 60 seconds x 12 times and intervals with cooling for 3 minutes. After the reaction, the mixture was cooled to room temperature and added 20 mL of ethyl acetate. Then, RO water was added, and the pH was adjusted to **9–10** with 5% NaOH $_{(aq)}$. Separation of ethyl acetate fraction and water fraction was conducted. The ethyl acetate fraction was washed with brine, dried with $MgSO_4$, and evaporated to result in ethyl cinnamate derivatives (**5–7**).

Ethyl cinnamate (**5**). Colourless liquid. ^{1}H NMR (700 MHz, Chloroform-*d*) δ 7.68 (d, *J* = 15.9 Hz, 1H), 7.51 (dd, *J* = 6.7, 3.1 Hz, 2H), 7.39 – 7.35 (m, 3H), 6.43 (d, *J* = 16.0 Hz, 1H), 4.26 (q, *J* = 7.2 Hz, 2H), 1.33 (t, *J* = 7.2 Hz, 3H) ppm. ^{13}C NMR (176 MHz, $CDCl_3$) δ 167.0, 144.6, 134.4, 130.2, 128.9, 128.0, 118.3, 60.5, 14.3 ppm. $C_9H_7O^+$ calculated *m/z* 131.05 and found *m/z* 131.10 (100%).

Ethyl 4-chlorocinnamate (**6**). Colourless liquid. ^{1}H NMR (700 MHz, Chloroform-*d*) δ 7.62 (d, *J* = 16.0 Hz, 1H), 7.44 (d, *J* = 8.4 Hz, 2H), 7.35 (d, *J* = 8.4 Hz, 2H), 6.40 (d, *J* = 16.1 Hz, 1H), 4.26 (q, *J* = 7.2 Hz, 2H), 1.33 (t, *J* = 7.2 Hz, 3H) ppm. ^{13}C NMR (176 MHz, $CDCl_3$) δ 166.8, 143.2, 136.2, 133.1, 129.3, 129.3, 119.0, 60.7, 14.4 ppm. $C_9H_6ClO^+$ calculated *m/z* 165.10 and found *m/z* 165.00 (100%).

Ethyl 4-nitrocinnamate (**7**). Colourless crystal. ^{1}H NMR (700 MHz, Chloroform-*d*) δ 8.25 (d, *J* = 8.8 Hz, 1H), 7.71 (d, *J* = 16.2 Hz, 2H), 7.68 (d, *J* = 8.7 Hz, 2H), 6.56 (d, *J* = 16.1 Hz, 1H), 4.30 (q, *J* = 7.0 Hz, 2H), 1.36 (t, *J* = 7.2 Hz, 3H) ppm. ^{13}C NMR (176 MHz, $CDCl_3$) δ 166.2, 148.6, 141.7, 140.7, 128.7, 124.3, 122.7, 61.1, 14.4 ppm. $C_9H_6NO_3^+$ calculated *m/z* 176.03 and found *m/z* 176.10 (100%).

2.3 Isolation of Ethyl 4-Metoxycinnamate (9)

Isolation of ethyl 4-metoxycinnamate (**9**) was conducted according to literature (Tachrim, et al., 2022).

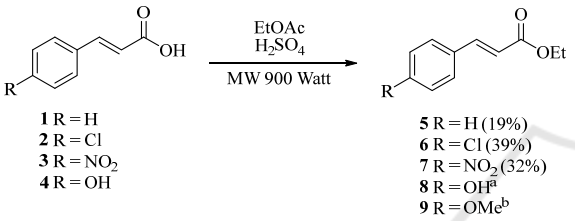
2.4 Brine Shrimp Lethality Assay

The procedure for BSLT followed the literature (Primahana, et al, 2015). Brine shrimps were hatched using brine shrimp eggs (*Artemia salina*) in a conical-shaped vessel, filled with filtered seawater. The number of dead and surviving nauplii in each tube was counted and recorded. LD_{50} values were determined from the best-fit line plotted concentration versus percentage lethality.

3 RESULT AND DISCUSSION

The reaction of cinnamic acid derivatives (**1**–**4**) with ethyl acetate and H_2SO_4 via microwave irradiation can be conducted without a reflux system within 60 seconds of irradiation time with 3 minutes cooling process. This interval is utilized for the prevention of further ethyl acetate evaporation due to the rapid high-temperature condition offered by microwave irradiation. The resulted ethyl cinnamate derivatives (**5**–**7**) resulted in moderate yield from the correspondent cinnamic acid (**1**), *p*-chloro (**2**), and *p*-nitro (**3**), respectively (Scheme 1). As for *p*-hydroxy (**4**, Scheme 1), after quenching the reaction mixture, no ethyl 4-hydroxycinnamate (**8**, Scheme 1) is isolated. Compound **4** might need a longer reaction time to conduct with the ethyl acetate system, since this compound has low solubility towards ethyl acetate and found up until 10 minutes of irradiation it cannot be dissolved completely.

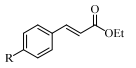
The conventional method of refluxing cinnamic acid derivatives with ethanol for the synthesis of ethyl ester derivatives (**5**–**7**) was also tested and showed a higher yield (60–80%) with 5 hour reaction time. In comparing these results with the current method, microwave irradiation is relatively more efficient due to its faster reaction time. Herein, the ethyl acetate utilization in sulfuric acid can offer esterification of semi-polar compounds of cinnamic acid derivatives (**1**–**3**) into its ethyl ester derivatives (**5**–**7**). This method can be used as an alternative to direct ethanol utilization (Fisher esterification) in which ethanol can be formed from the hydrolysis of ethyl acetate (Jaques, 1971; Vinnik and Librovich, 1975) under high temperature by microwave irradiation.



Scheme 1: Synthesis of ethyl cinnamate derivatives via microwave irradiation of cinnamic acids with ethyl acetate. The bracket showed an isolated yield. [a]No ethyl 4-hydroxycinnamate is isolated. [b]Ethyl 4-methoxycinnamate is isolated from rhizomes of *K. galanga* according to literature (Tachrim, et al., 2022).

The result of brine shrimp assay lethality assay of synthesized ethyl cinnamate derivatives **5**–**7** and ethyl 4-methoxycinnamate **9** isolated from the rhizome of *K. galanga* are shown in Table 1 and Fig. 1. Based on these results, the toxicity level of all tested compound is **6** > **9** > **5** > **7**. The synthesized ethyl cinnamate **5** and *p-nitro* **7** showed moderate toxicity compared with the ethyl 4-methoxycinnamate **9** isolated from the rhizome of *K. galanga*. *p*-Chloro **6** showed high toxicity among all tested compounds with LC_{50} of 1.29 µg/mL and has highest % mortality on 10 ppm. For comparison, the reported brine shrimp lethality assay on crude CH_2Cl_2 extract of *K. galanga* with the major compound of ethyl cinnamate and ethyl 4-methoxycinnamate showed almost 100% mortality can be achieved at a concentration of 10 ppm (Othman, et al., 2006). The previous brine shrimp lethality assay of methyl cinnamate also showed a moderate LC_{50} of ~120 µg/mL (Primahana, et al., 2015). Despite further study is needed to understand the structure correlation with the toxicity, the high toxicity of *p*-chloro **6** is due to the introduction of chloro as a substituent on the aromatic moiety. Moreover, this compound has the potential to be used for the study of its bioactivity in the future.

Table 1: Brine shrimp lethality assay of synthesized ethyl cinnamate derivatives.

	Mortality (%)				LC_{50} (ppm)
	10 ppm	100 ppm	500 ppm	1000 ppm	
5 (R = H)	0	6	100	100	234.42
6 (R = Cl)	61	98	100	100	1.29
7 (R = NO_2)	4	17	74	93	301.99
9 (R = OMe)	5	17	52	82	173.78

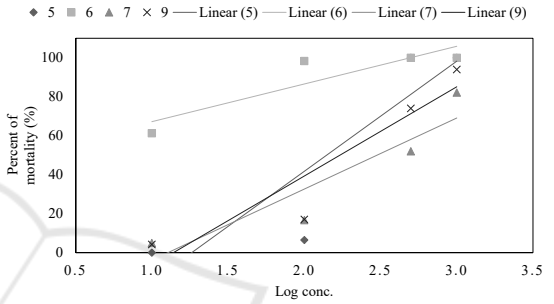


Figure 1: Effect of synthesized ethyl cinnamate derivatives **5**–**7** and **9** on brine shrimp nauplii.

4 CONCLUSIONS

The reaction of cinnamic acid derivatives (**1–3**) with ethyl acetate and H_2SO_4 via microwave irradiation can be used as an alternate method for the synthesis of their representative ethyl esters (**5–7**). Among the synthesized ethyl cinnamate derivatives 5–7 and ethyl 4-methoxycinnamate 9 isolated from the rhizome of K. galanga, ethyl *p*-chloro cinnamate 6 showed the highest toxicity level with LC_{50} of 1.29 µg/mL. The brine shrimp lethality assay tested in this study can provide the toxicity data comparison of several ethyl esters of cinnamic acid derivatives before in vivo study and contribute to the preliminary study of drug development.

ACKNOWLEDGEMENTS

RMA was supported by the Postdoctoral Fellowship National Research and Innovation Agency, Indonesia. NU thanks to Research Assistant Fellowship National Research and Innovation Agency, Indonesia.

REFERENCES

Anthony, P. C., Eseyin, O. A., Attih, E., Johnson, E., Ebong, A., Effiong, A. E., 2022. Synthesis of Some Esters of Cinnamic Acid and Evaluation of Their in Vitro Antidiabetic and Antioxidant Properties. 2022. *Trop. J. Pharm. Res. 21* (1), 131–136.

Dash, P. R., Nasrin, M., Ali, M. S., 2014. In Vivo Cytotoxic and In Vitro Antibacterial Activities of *Kaempferia galanga*. *J. Pharmacogn and Phytochem. 3* (1), 172–177.

Elshamy, A. I., Mohamed, T. A., Essa, A. F., Abd-ElGawad, A. M., Alqahtani, A. S., Shahat, A. A., Yoneyama, T., Farrag, A. R. H., Noji, M., El-Seedi, H. R., Umeyama, A., Paré, P. W., Hegazy, M. F., 2019. Recent Advances in *Kaempferia* Phytochemistry and Biological Activity: A Comprehensive Review. *Nutrients 11* (10), 2396.

Hoz, A. D., Diaz-Ortiz, A., Moreno, A., 2005. Selectivity in Organic Synthesis under Microwave Irradiation. *Curr. Org. Chem. 8* (10), 903–918.

Jaques, D., 1971. Hydrolysis of Ethyl Acetate in Concentrated Sulfuric Acid. A Group Experiment for Advanced Students. *J. Chem. Educ. 48* (9), 623.

Komala, I., Supandi, Nurhasni, Betha, O. S., Putri, E., Mufidah, S., Awaludin, M. F., Fahmi, M., Reza, M., Indriyani, N. P., 2018. Structure-Activity Relationship Study on the Ethyl *p*-Methoxycinnamate as an Anti-Inflammatory Agent. *Indones. J. Chem. 18* (1), 60–65.

Michael, A. S., Thompson, C. G., Abramovitz, M., 1956. Artemia Salina as a Test Organism for Bioassay. *Science 123* (3194), 464–464.

Primahana, G., Ernawati, T., Dewi, N. L. P., Dwiyatmi, I. D., Darmawan, A., Hanafi, M., 2015. Synthesis of 2-Allylphenyl Cinnamate and Brine Shrimp Lethality Test Activity Evaluation. *Procedia Chem. 16*, 694–699.

Othman, R., Ibrahim, H., Mohd, M. A., Mustafa, M. R., Awang, K., 2006. Bioassay-Guided Isolation of a Vasorelaxant Active Compound from *Kaempferia galanga* L. *Phytomedicine 13* (1–2), 61–66.

Tachrim, Z. P., Kurniawan, K., Andreani, A. S., Sundowo, A., Ernawati, T., Darmawan, A., Dewi, R. T., 2022. Insight Study of Trans-Ethyl *4*-Methoxycinnamate Isolation and Hydrolysis. *AIP Conf. Proc. 2493*, 070014–15.

Umar, M. I., Asmawi, M. Z., Sadikun, A., Majid, A. M. S. A., Al-Suede, F. S. R., Hassan, L. E. A., Altaf, R., Ahamed, M. B. K., 2014. Ethyl-*p*-Methoxycinnamate Isolated from *Kaempferia galanga* Inhibits Inflammation by Suppressing Interleukin- 1, Tumor Necrosis Factor-α, and Angiogenesis by Blocking Endothelial Functions. *Clinics 69* (2), 134–144.

Vinnik, M. I., Librovich, N. B., 1975. Mechanism of the Hydrolysis of Ethyl Acetate in Aqueous Solutions of Strong Acids. *Bull. Acad. Sci. USSR Div. Chem. Sci. 24* (10), 2097–2102.

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