

# Valorization of Keting fish (*Mystus nigriceps*) viscera using papain for antioxidant protein hydrolysate production

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**Abstract.** Underutilized viscera from keting fish (*Mystus nigriceps*) processing in Kenjeran, Surabaya offer opportunities for resource recovery and pollution prevention. This study aimed to optimize the hydrolysis conditions of keting viscera using papain and to evaluate its potential as a source of antioxidant protein hydrolysate. The optimal hydrolysis conditions were determined based on the most effective scavenging activity of 2,2-diphenyl-1-picrylhydrazil (DPPH) radical. Viscera were hydrolyzed under various conditions, including four enzyme concentration levels (0, 1, 3, and 5%) and four hydrolysis durations (1, 2, 4, and 6 hours). Enzyme concentration, hydrolysis duration, and their interaction showed a significant influence on antioxidant activity ( $p < 0.05$ ). Hydrolysate was produced under optimal conditions with a 5% papain solution applied for six hours. This resulted in a product with strong antioxidant properties, as measured by its  $IC_{50}$  value of 1.22 mg/mL against DPPH radicals. The proximate analysis of the hydrolysate showed the protein content was 30.04% and distributed its molecular weight in the range of 68 – 134 kDa and  $\leq 10$  kDa. This research demonstrates the feasibility of valorizing fish processing waste into a valuable product with prospective uses in the functional food, nutraceutical and pharmaceutical fields.

## 1. Introduction

Fish processing produces considerable quantity of by-products, including bones, viscera, skin, scale, and heads, which are mostly discarded [1, 2]. Improper disposal of such processing by-products might cause serious environmental issues due to their high organic load, creating favorable conditions for disease outbreaks. The organic matter present in fish waste contributes to the eutrophication of water bodies, thus depleting the oxygen levels in water and harming aquatic ecosystems [3].

The repurposing of underutilized fish by-products supports the principles of a circular economy, offering a sustainable pathway to high-value products and economic benefits. The valorization of fish by-products into bioactive peptides provides a sustainable route to high-value products [4]. Hydrolysis using enzyme is a green technology for producing transforming these by-products into value-added products, for example, fish protein hydrolysate (FPH), which possess valuable bioactivities, including antioxidant properties [5, 6]. FPH have attracted



substantial interest recently given its prospective uses in food, pharmaceutical, and cosmetic industries. FPH is known to be rich in antioxidants, which can help in combating oxidative stress, a major cause of many chronic diseases [6, 7]. However, FPH production technology needs to focus on the development of the hydrolysis process to achieve higher yields, higher degree of hydrolysis and bioactivity. A number of investigations have focussed on the FPH production from diverse fish species using different enzymes. The hydrolysis of *Acipenser sinensis* using 3% papain for six hours and 3.5% alcalase for six hours [8], *Channa striata* using 3% papain for three hours [9], and Indian mackerel using 1% papain for six hours [10] resulted in FPH with antioxidant activity. Based on these studies, different fish species and enzymes have varying optimal hydrolysis durations and enzyme concentrations.

Keting fish (*Mystus nigriceps*) is a commonly consumed fish species in Surabaya, Indonesia. A popular processing method, smoking, generates significant amounts of fish processing by-products, including viscera. This study focused on optimizing the enzymatic hydrolysis of keting fish viscera with papain to produce high-quality FPH with high antioxidant activity. By investigating the impact of hydrolysis duration and papain concentration on FPH yield and antioxidant activity, this study contributes to the sustainable utilization of underutilized fish by-products and explores avenues for developing novel health-promoting food elements.

## 2. Materials and Methods

### 2.1 Viscera collection and processing

The viscera of *M. nigriceps* were obtained from a smoked fish producer in Kenjeran, Surabaya. The viscera were stored in an icebox to maintain freshness during transportation. The viscera underwent cleaning and homogenization in sterile distilled water (DW; 1:1, w/v). The homogenized viscera were centrifuged to obtain pellet from supernatant. The viscera pellet was stored in a freezer prior to further experiments.

### 2.2 Papain-assisted hydrolysis of viscera

The hydrolysis conditions were optimized by varying the papain concentration (0, 1, 3, and 5%) and hydrolysis duration (1, 2, 4, and 6 hours). Hydrolysis was carried out at 60 °C in an incubator with a stirring speed of 225 rpm. The hydrolysate was centrifuged, and the supernatant was collected. The enzymatic activity was terminated by inactivating the papain in a waterbath at 95 °C for 15 minutes.

### 2.3 Evaluation of antioxidant by 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging activity

The hydrolysate's antioxidant activity was evaluated using the DPPH radical scavenging assay [11, 12]. Hydrolysate diluted to 5% in DW and mixed with DPPH reagent in a 1:1 ratio (v/v) in a microplate (96-well). The microplate covered with foil and maintained at room temperature for 30 min, followed by measurement of the absorbance at 517 nm (A) using an ELISA reader. The antioxidant activity was calculated according to the equation below:

$$\text{DPPH radical-scavenging activity (\%)} = \frac{(A_{\text{control}} - A_{\text{blank 1}}) - (A_{\text{sample}} - A_{\text{blank 2}})}{(A_{\text{control}} - A_{\text{blank 1}})} \times 100$$

Blank<sub>1</sub> is DW as a control blank; and blank<sub>2</sub> is sample without DPPH (replaced by methanol) as a sample blank. The hydrolysate concentration required to achieve 50% inhibition (IC<sub>50</sub>) of the DPPH radical was determined by plotting the concentration (X-axis) against the percentage of

inhibition (Y-axis). The hydrolysate used in  $IC_{50}$  measurements was oven-dried at 50°C for 48 hours, powdered, and dissolved in DW to obtain various concentrations (0.1-2.0 mg/mL).

#### *2.4 The measurement of hydrolysate proximate composition*

Moisture, ash, lipid, protein, and carbohydrate contents were measured as part of the proximate composition. Moisture content was measured gravimetrically after oven-dried at 100 °C. Ash content was measured gravimetrically after being processed at 550 °C in a furnace [13]. Lipid content was measured gravimetrically following extraction with a 2:1 ratio (v/v) of methanol and chloroform and subsequent solvent evaporation [14]. Protein content was analyzed using the Kjeldahl method after digestion with sulfuric acid at high temperature [15] Carbohydrate content was determined using the by-difference method by subtracting the moisture, ash, lipid, and protein analysis results from 100% [16].

#### *2.5 Determination of Hydrolysate Molecular Weight using SDS-PAGE*

The hydrolysate's molecular weight distribution was examined with SDS-PAGE [11]. Hydrolysate was dissolved in SDS-PAGE buffer and heated to denature the proteins. The hydrolysate sample was then loaded onto a gel and separated by electrophoresis. The protein bands were treated for an hour with Brilliant Blue R-250 and then underwent a destaining process for three hours. Protein bands were visualized with blue light transilluminator and compared with the protein molecular weight marker to determine their molecular weights.

#### *2.6 Statistical analysis*

Statistical evaluations were performed to analyze the data for DPPH radical-scavenging activity and hydrolysis yield. Normal data distribution was checked using the Shapiro-Wilk test, and homogeneity of variance was checked using Levene's test. Data conforming to these assumptions were analyzed using a two-way ANOVA with a significance level set at  $\alpha = 0.05$  to evaluate the effects of enzyme concentration, hydrolysis duration, and their interaction on DPPH radical scavenging activity. Tukey's honestly significant difference test was used for post hoc comparisons. The proximate composition was analyzed using descriptive statistics to calculate the mean from three replicates.

### **3. Results and discussion**

#### *3.1 Antioxidant activity of papain hydrolyzed-fish viscera*

This study aimed to evaluate the best conditions for producing fish viscera protein hydrolysates with enhanced DPPH radical scavenging activity. Hydrolysates generated under various hydrolysis conditions exhibited differing levels of antioxidant activity, as presented in Table 1. Antioxidant activity showed significant differences with increasing enzyme concentration and hydrolysis duration ( $p < 0.05$ ). Tukey's post hoc test ( $p < 0.05$ ) indicated that hydrolysis of viscera with 5% papain for 6 hours resulted in the highest antioxidant activity, reaching  $69.57 \pm 0.30\%$ . The rise in antioxidant activity over the hydrolysis duration is attributed to the generation of antioxidant peptides. Enzymatic hydrolysis cleaved the proteins in kating viscera into smaller peptides, resulting in lower molecular weights. Peptides with lower molecular weights are recognized for their enhanced antioxidant effectiveness [17]. However, prolonged hydrolysis can reduce activity by breaking bioactive peptides into non-bioactive fragments [18], highlighting the importance of optimizing hydrolysis duration. The enhanced activity is also linked to the production of hydrophobic peptides during hydrolysis [19]. Ma et al. [20] reported that bioactive peptides from tilapia skin contain hydrophobic amino acids proline, alanine, and glycine that

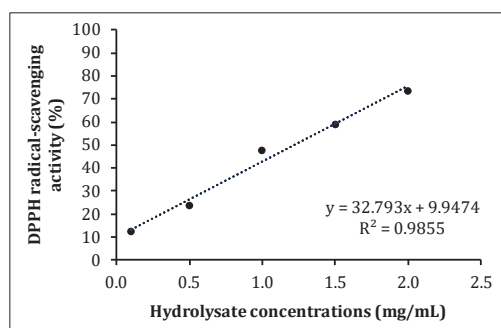
showed scavenging interactions with the free radicals. Higher enzyme concentrations increase the yield of bioactive peptides [21]. The hydrolysate produced under optimal conditions in this study showed a DPPH radical-scavenging activity of 73.80% at a concentration of 2 mg/mL. This value aligns to those reported by Jemil et al. [22] for stingray hydrolysate produced using *Bacillus subtilis* enzymes (75.00% at 6 mg/mL), and by Ktari et al. [23] for zebrafish hydrolysates produced using fish protease (76.56% at 6 mg/mL).

**Table 1.** The effect of various papain concentration (%) and hydrolysis duration (h) on the antioxidant activity (% DPPH radical scavenging) of the hydrolysate.

Papain concentration (%)	Hydrolysis duration (h)			
	1	2	4	6
0	47.13 ± 0.51 <sup>ab</sup>	44.69 ± 0.13 <sup>a</sup>	48.85 ± 2.18 <sup>ab</sup>	50.94 ± 0.26 <sup>abc</sup>
1	45.61 ± 1.79 <sup>a</sup>	46.53 ± 1.81 <sup>ab</sup>	51.64 ± 0.26 <sup>abc</sup>	53.74 ± 0.39 <sup>bcd</sup>
3	51.29 ± 3.39 <sup>abc</sup>	53.31 ± 3.20 <sup>bcd</sup>	60.19 ± 0.70 <sup>de</sup>	60.88 ± 1.39 <sup>def</sup>
5	57.68 ± 3.65 <sup>cde</sup>	58.87 ± 2.53 <sup>cde</sup>	65.93 ± 2.24 <sup>ef</sup>	69.57 ± 0.30 <sup>f</sup>

The DPPH radical scavenging activity values provided (mean ± standard deviation) were determined using a 5% diluted liquid hydrolysate. Tukey's HSD test reveals significant differences ( $p < 0.05$ ) within the combinations of papain concentration and hydrolysis duration, as shown by different letters (a to f) in the columns.

The optimal hydrolysate, produced using 5% papain for 6 hours, exhibited a lower  $IC_{50}$  value (1.22 mg/mL; Figure 1) compared to scalloped hammerhead muscle hydrolysates (3.06 mg/mL) reported by Luo et al. [24], but higher than those smooth hound muscle (0.60 mg/mL) and stone fish tissue (0.49 mg/mL) reported by Bougatef et al. [25] and Bordbar et al. [26], respectively. The lower  $IC_{50}$  values indicating greater efficacy [27]. These findings suggest that *M. nigriceps* viscera hydrolysates possess promising antioxidant potential. Variations in fish species, tissue, enzyme type, concentration, and hydrolysis duration can influence antioxidant activity. Different fish species have varying protein and amino acid compositions, leading to diverse bioactive peptide profiles. The bioactivity of hydrolysates is partly determined by the specificity of the enzyme used [28].



**Figure 1.** The DPPH radical-scavenging activity (%) of keting fish protein hydrolysate was evaluated at various concentrations (mg/mL). To determine the  $IC_{50}$  value, the concentration required to inhibit 50% of DPPH radicals, linear regression analysis was employed.

### 3.2 Yield of the papain hydrolyzed-fish viscera

Hydrolysate yield is the percentage of dry product relative to initial substrate, reflects hydrolysis efficiency. High yield values signify an efficient conversion of kating visceral protein into peptides by papain. However, the enzyme concentration significantly influenced yield ( $p < 0.05$ ), while hydrolysis duration did not. Enzyme concentration affects hydrolysate yield because more enzyme means more active sites available to cleave proteins, thus increasing hydrolysis efficiency [29]. At the optimal conditions (5% papain, 6 hours), one kilogram of *M. nigriceps* viscera yielded approximately 97.1 grams of dried hydrolysate (9.71%; Table 2). This compares favorably to previous studies [30, 31], suggesting that hydrolysate yield varies with enzyme type, concentration, and fish part.

**Table 2.** The effect of various papain concentration (%) and hydrolysis duration (h) on the yield (%) of the hydrolysate.

Papain concentration (%)	Hydrolysis duration (h)			
	1	2	4	6
0	6.91 ± 0.66	7.02 ± 0.08	6.31 ± 0.14	5.22 ± 0.08
1	8.07 ± 0.54	7.58 ± 0.46	6.72 ± 0.19	5.28 ± 0.21
3	12.02 ± 0.12	11.16 ± 0.54	9.38 ± 0.74	7.68 ± 0.17
5	15.36 ± 0.21	14.09 ± 0.42	12.27 ± 0.69	9.71 ± 0.34

### 3.3 Proximate composition of the raw fish viscera and papain hydrolyzed-fish viscera

Proximate analysis of viscera hydrolysate from *Mystus nigriceps* revealed a protein content of 30.04%, lipid content of 0.78%, and moisture content of 22.14% (Table 3). The high protein content and antioxidant bioactivity of this hydrolysate suggest its potential applications in various fields, including human food, dietary supplements, and animal feed [32 – 34]. The relatively high moisture content indicates a need for further drying to enhance product stability [35]. Comparison with previous studies [33, 36 – 38] showed that the composition of hydrolysates can vary depending on the type of fish and processing methods. The FPH produced in this study exhibited a lower lipid content compared to the FPH from snapper fish waste meat hydrolysate (4.05%) reported by Prayudi et al. [39]. This difference could be explained by the higher lipid content in the raw material used in this study. The centrifugation process is also effectively reduced the lipid content, minimizing the risk of lipid oxidation in the hydrolysate [40].

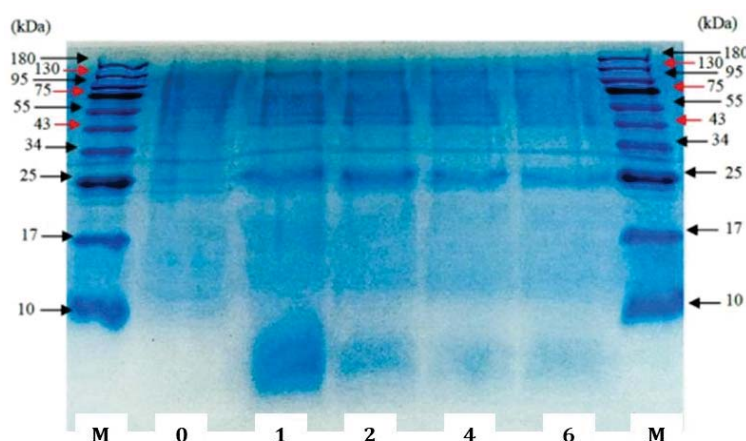
**Table 3.** The proximate composition of the raw viscera, liquid and dried hydrolysates.

Proximate composition	Content (%)		
	Raw viscera	Liquid hydrolysate	Dried hydrolysate
Protein	7.60 ± 1.91	2.63 ± 0.16	30.04 ± 0.72
Lipid	0.19 ± 0.02	0.05 ± 0.03	0.78 ± 0.03
Ash	3.00 ± 0.01	0.18 ± 0.01	2.81 ± 0.07
Water	83.93 ± 0.01	93.75 ± 0.19	22.14 ± 0.53
Carbohydrate	5.27 ± 1.88	3.39 ± 0.00	44.24 ± 0.09

The proximate composition of the hydrolysate under optimal hydrolysis conditions (5% papain and hydrolysis for 6 hours). The dried hydrolysate is obtained by drying the liquid hydrolysate in an oven at 50°C for 48 hours.

### 3.4 The molecular weight distribution of the papain hydrolyzed-fish viscera

SDS-PAGE results revealed that papain successfully hydrolyzed *M. nigriceps* viscera proteins into smaller peptides over time (Figure 2). During the hydrolysis process, enzymatic cleavage of the protein can be observed. This is evidenced by the faint bands appearing on SDS-PAGE, which indicate the fragments resulting from enzymatic action [41]. Protein bands with high molecular weights (approximately 43 - 130 kDa) became thinner, while bands with low molecular weights (less than 10 kDa) became thicker. This indicates an increase in the amount of small peptides due to papain enzyme activity. These findings are consistent with previous studies [42 – 44] reporting that protein hydrolysates generally contain small peptides. Small peptides have the potential to exhibit high biological activities, such as antioxidant effects, due to their easy interaction with free radicals. Hydrolysis produces lower molecular weight peptides that are enriched in hydrophobic amino acids. These peptides can interact with free radicals via hydrogen bonding and hydrophobic interactions [20]. Subsequent study should focus on the purification and sequencing of antioxidant peptides to improve their efficacy and safety, and to gain a better understanding of their functional mechanisms.



**Figure 2.** The effect of 5% papain hydrolysis on the molecular weight of *M. nigriceps* viscera. Molecular weights were observed using SDS-PAGE at 0, 1, 2, 4, and 6 hours of hydrolysis at 60°C. M represents the standard marker with a known molecular weight (kDa).

## 4. Conclusion

This study demonstrates that enzymatic hydrolysis of kiting fish viscera using 5% papain for 6 hours is the optimal condition to produce hydrolysate with high antioxidant activity. The resulting hydrolysate is rich in low molecular weight proteins, which could be a valuable source of bioactive peptides. This indicates the great potential of hydrolysates as functional ingredients in various products, such as food, pharmaceuticals, and cosmetics. However, further investigation is necessary to define the active peptides and ensure the safety and stability of the hydrolysate before commercial application. These findings open new opportunities for the conversion of fishery waste into economically valuable products with health benefits.



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## Preface: The International Conference and Workshop in conjunction with the 9<sup>th</sup> Indonesia Biotechnology Conference (IBC)

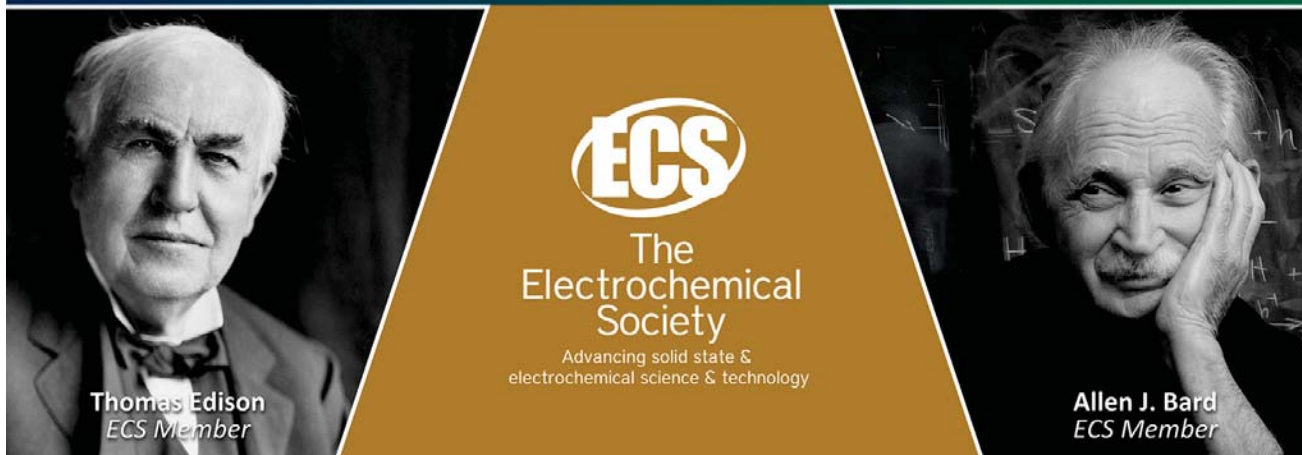
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## **Preface: The International Conference and Workshop in conjunction with the 9<sup>th</sup> Indonesia Biotechnology Conference (IBC)**

The International Conference and Workshop in conjunction with the 9<sup>th</sup> Indonesia Biotechnology Conference (IBC), was held by Biotechnology Study Program of Universitas Negeri Malang and Indonesian Biotechnology Consortium, in collaboration with National Research and Innovation Agency of Indonesia (BRIN) and Faculty of Biotechnology, Universitas Surabaya on 11-14 September 2024 at the Santika Premiere Hotel, Malang. The conference is dedicated to providing a forum to build network, exchange ideas about bioscience and biotechnology for addressing the challenges in sustainable developments, as well as to inspire young academics, scientists and students who are interested in biotechnology (including the new omics technology), biosciences and bioengineering and integrated approaches, and encourage ideas dissemination in these fields.

The joint conference's theme was "Accelerate Green Industrial Innovation Trough Biotechnology Towards the Golden Indonesia 2045 Vision" to drive crucial discussions on the advancement of biotechnology and life sciences. This conference serves as a vital platform for researchers, educators, policymakers, and industry leaders need to share their insights, innovations, and experiences in the fields of biotechnology, biology, bioinformatics, biochemistry, environmental sustainability, renewable energy, and health issues. This conference resonates deeply with the commitment to fostering knowledge that not only meets the demands of the present especially in the realm of biotechnology and life sciences but also ensures a sustainable future for all. This is in line with Sustainable Developments Goals point 9 and 17, which are Industry, Innovation, and Infrastructure as well as Partnerships for the Goals. This conference also serves as a beacon of intellectual exchange, where ideas are shared, collaborations are formed, and solutions to global challenges are sought.

The objectives of the conference were: 1) to bring together participants, experts and stakeholders to examine ideas and solutions for maintaining, caring and protecting biodiversity for future sustainable development for human beings, especially in Indonesia, 2) to integrate ideas and inputs from academics from various discipline background to achieve prosperous sustainable biodiversity, 3) to provide a chance of networking between academics and practitioners dealing with biodiversity, 4) to exchange research results on sustainable use as well as conservation of biodiversity, 5) to increase capacity building of young researchers in exploring biodiversity for human health and wellness, and 6) to broaden networking of the participants and to produce a joint research.

The benefit of this conference was creating synergy between national and international academics and institutions with various discipline backgrounds, government bodies, and stakeholders dealing with biodiversity for supporting sustainable development and future community prosperity. The conference and workshop are also a premier interdisciplinary forum for life scientists, engineers, and practitioners to present their latest research results, ideas, developments, and applications in relevant areas.

The joint conference was attended by academics, researchers and practitioners within broad areas of science and technology such as agriculture, forestry, biotechnology and environment, medicine, biology, veterinary, and social sciences. The presented materials have undergone peer review processes and only qualified papers were selected. Furthermore, all papers were subjected to double-blind peer-review and expected to meet the scientific criteria of significance and academic excellence in IOP Proceeding Conference Series.

This two-day conference on 11-12 September 2024 featured research presentations and was closed with discussions with experts and participants to sum up the common conclusion. This occasion was followed by an excursion to Mount Bromo in Malang and variety of workshops (Halal Analysis, Bioinformatics in Biomarker Analysis and Drug Discovery, GMO Analysis, as well as Plant Tissue



Culture) for selected participants on 13-14 September 2024. The first day of the conference consisted of nine keynote speakers' around 25 minutes presentations divided into three plenary sessions. The second day was organized for 6 keynote speakers, followed by parallel sessions which were divided into four offline rooms and five online rooms. The speakers are listed below:

1. Prof. Dato' Dr. Amirul Al-Ashraf Abdullah (Universiti Sains Malaysia)
2. Prof. Dr. Chiaki Ogino (Kobe University, Japan)
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9. Dr. Kanyaratt Supaibulwatana (Mahidol University, Thailand)
10. Prof. Mariana Wahjudi, Ph.D (Universitas Surabaya, Indonesia)
11. Prof. Dr. Suharti, S.Pd., M.Si (State University of Malang, Indonesia)
12. Prof. Tri Agus Siswoyo, M.agr., Ph.D (Universitas Jember, Indonesia)
13. Rezzy Eko Caraka, Ph.D (National Research and Innovation Agency)
14. Dr. Adulsman Sukkaew (Vice President of the Southern Border Researchers Association, Thailand)
15. Dr. Sanghapal D. Sawant (CSIR-Indian Institute of Integrative Medicine, Jammu and SRTM University, India)

We would like to express our sincere gratitude to all speakers, presenters, and all participants for their contributions to this conference and workshop. We would also like to express our appreciation for the generosity of our sponsors who supported this conference:

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Lastly, special thanks to all steering committee, reviewers, and committee members for their great work and contributions to the conference, publication, and workshop.

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