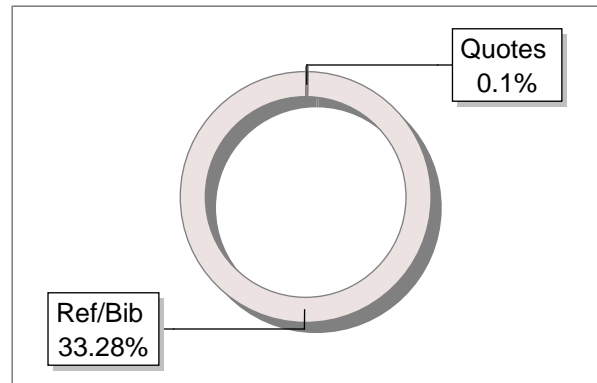
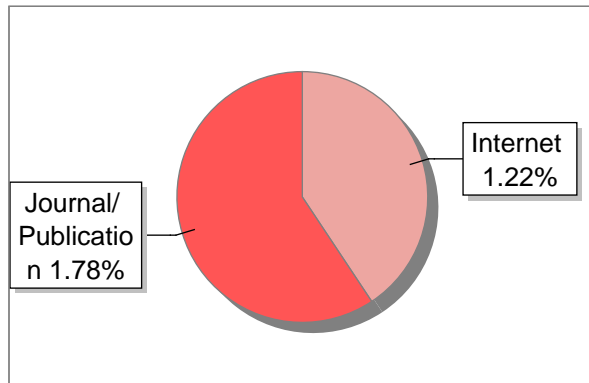
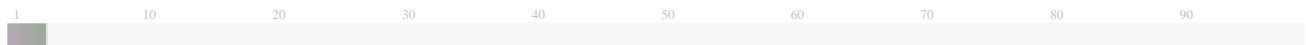


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Development of detection method for carcinogenic compounds using radish seeds (*Raphanus sativus* L.) via measuring the alkaline phosphatase enzyme activity

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ABSTRACT

Convincing data regarding carcinogenic substances is very useful for handling materials correctly to avoid exposure to cancer-triggering substances in the body. To collect as much carcinogen data as possible, low-requirement carcinogen detection setups need to be developed. In this study, the first steps in the development of carcinogen detection through functional protein expression assessment are reported. Hydrazine sulfate, one of the well-known carcinogens chosen as a model material, was tested on germination of *Raphanus sativus* L., with various concentrations, namely 0 (control), 1, 10, and 100 mM. This plant was selected due to its characteristics showing a stable germination rate over a wide range of temperatures. Within a predetermined germination time, seeds and/or sprouts were observed and germination rate, protein concentration, and alkaline phosphatase activity were measured. The results showed that hydrazine sulfate slowed seed germination, and increased 72-h protein concentration but decreased alkaline phosphatase activity in the control group. This suggests that changes in alkaline phosphatase activity in seeds during germination have the potential to be a marker for cancer. In this research, it can be concluded that the method used can be applied as a first step to assess the carcinogenicity of a compound.

Keywords: Hydrazine sulfate, carcinogen, germination, *Raphanus sativus*, alkaline phosphatase

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INTRODUCTION

Cancer is the second-leading disease causing death in the world (Dattani et al., 2023). Various factors can influence the incidence of cancer including diet, radiation, genetics, and lifestyle. Carcinogenic compounds, substances that trigger cancer, can originate from food and the environment such as air or industrial materials as contaminants in the manufacturing process. In line with the motto "Prevention is better than cure", minimizing contact with carcinogenic substances is the first step to avoid the emergence of cancer. Identification of the carcinogenic activity of suspected substances should be made known to health authorities.

In addition to molecular structure analysis (Chung et al., 2023) and epidemiological studies (Gallagher et al., 2023; Mattiuzzi & Lippi, 2019), identification of the carcinogenic potential of substances has been conducted by introducing the substance of interest to short-term or long-term testing. In short-term *in vitro* testing, a suspected carcinogen is introduced into cultures of mammalian cells as well as unicellular organisms, followed by the identification of characteristic physiological and/or genetic transformations (Li et al., 2019). Several methods were developed for *in vivo* short-term carcinogenic compound detection. These include studying the suppression of sebaceous glands (Zouboulis et al., 2022), degranulation of reticulum endoplasmic of rat liver (Mathankumar et al., 2015), tissue reaction to subcutaneous implant (Tazawa et al., 2007), and recently, isolating and culturing macrophages from animal models exposed to a suspected carcinogenic substance (Schlatterer et al., 2022). A full animal model is also used for long-term testing. Briefly, rodents are exposed to the target substance orally or by other routes for quite a long time, almost for the rest of their lives. Abnormal tissue growth, namely neoplastic lesions, is a marker of positive results. At the same time, mechanistic studies of carcinogenesis are traced by evaluating cellular changes and biochemical measurements (EMA, 2018).

Both previously described carcinogen detection methods have limitations, especially regarding rigorous protocols, and cell culture models that require maintenance of sterility to avoid the growth of atmospheric microorganisms in the medium. In addition, cultural media are relatively expensive, animal models must also be carried out according to strict ethics. Moreover, analyzing neoplastic lesions is not a trivial and inexpensive task. Therefore, this study aimed to develop a new method to detect carcinogenic compounds based on the expression of specific enzyme related to cancerous tissue growth. Cancer tissue shows different characteristics compared to normal tissue, including enzyme expression. In cancer, some enzymes can increase or decrease their activity depending on the type of cancer. Comprehensive discussions regarding functional protein expression in mammals with cancer were also carried out in several experiments (Nilsson et al., 2014; Pozzi et al., 2023) and the same also applies to unicellular organisms, such as bacteria and yeast (Eki, 2018; Ziger, 2019). In this research, the influence of carcinogens on the germination of plant seeds, namely *Raphanus sativus* L., was used as a first step in developing a method for detecting carcinogenic substances. The advantage of selecting this plant is that its germination is relatively unaffected by the variation in environmental temperature over the range of 20 to 40°C (Kaya, 2023).

To develop a new detection method, one should select a model of a substance showing an established carcinogenic activity, such as hydrazine sulfate. Hydrazine sulfate, which is commonly used in metal refining analysis, was chosen since it induces lung, colorectal, and liver cancer (Directorate-General for Employment et al., 2017; Morris et al., 2015; Ritz et al., 2006; Venkatachalam et al., 2020). The mechanism of carcinogenesis associated with hydrazine is alkylation, especially at guanine nucleobases in DNA which causes mutations (Specer & Kisby, 2021). Moreover, it is uncovered that biotransformation also plays a role in the toxicity of hydrazine (Sinha & Mason, 2014; Venkatachalam et al., 2020).

Another aspect in the development of the carcinogen detection method is choosing the marker. An acknowledged enzyme available in living organisms, including in plants, is phosphatase. Alkaline phosphatase, a biocatalyst of phosphate hydrolysis in base condition, increases expression during germination (Desai et al., 2023). Since in mammals changes in this enzyme indicate the growth of cancerous tissue (Ren et al., 2015), it is likely that the same situation also occurs in plants. Within this research, germination of radish (*Raphanus sativus* L.) seeds containing hydrazine sulfate was carried out, followed by enzyme collection and protein concentration calculations. Furthermore, enzyme activity

measurements were carried out to analyze whether changes in base (alkaline) phosphatase activity due to the presence of hydrazine sulfate.

MATERIALS AND METHOD

Materials

Hydrazine sulfate, Folin-Ciocalteu reagent (Merck), Bovine serum albumin (BSA), trichloroacetic acid (TCA), Na- β -glycerophosphate, SnCl_2 , and ammonium molybdate were purchased from Sigma, Alfa Chemical, Yogyakarta. Radish seeds were obtained from the farm shop in Yogyakarta. Spectrophotometer UV-Vis (Genesys 5) was used in determining protein concentration and enzyme activity.

Methods

Germination of radish seeds

The germination of radish seeds was conducted by growing the radish seeds in Petri dishes at the time of 24, 48, and 72 hours of storage in a dark place at room temperature. The petri dishes were covered and the filter paper was put on both sides of the petri (Hussein & Joo, 2018; Kaya, 2023). There are two groups of experiments namely sample groups which consist of three levels of hydrazine sulfate concentrations namely 1, 10, and 100 mM, and the second group is the control group without the addition of hydrazine sulfate. At the end of each predetermined time of incubation, the number of germinated seeds was quantified and the rate of germination of each group and the decrease in germination of treatment groups were calculated based on equations 1 and 2, respectively.

$$\text{Germination rate (\%)} = \frac{\text{Germinated seeds number}}{\text{Total seeds number}} \times 100\% \quad (1)$$

$$\text{Decrease in germination (\%)} = \frac{G_c - G_t}{G_c} \times 100\% \quad (2)$$

in which G_c and G_t are the mean of germination rate of the control group and treatment group respectively.

Enzyme sample preparation

At each predetermined incubation time, the germinating radish seeds were harvested. Protein and enzyme extraction of the harvested sprouts was performed using roughly isotonic aqueous-based solvent simplified from a reference procedure (Habiba et al., 2021). Nine portions of 1% sodium chloride were added to the sprout in an insulated ceramic mortar in iced water, followed by hand crushing to obtain a homogenous suspension and to dissolve water-soluble substances including proteins. Centrifugation at 7000xg was applied for 45 min to the suspension to sediment undissolved material. The supernatant was collected for protein quantification and enzyme activity determination directly or after freeze-stored.

Determination of protein concentration

The protein concentration in the sample was quantified following the Lowry method (Lowry et al., 1951; Mæhre et al., 2018). In this method, 4 reagents were prepared, namely Lowry A reagent (2% of Na_2CO_3 in 0.1 N NaOH), Lowry B reagent (0.5% of CuSO_4 in 1% Na-tartrate), Lowry C reagent (a freshly prepared mixture of Lowry A reagent and Lowry B reagent in a portion of 50:1), and diluted Folin-Ciocalteu reagent (containing phosphomolybdate and phosphotungstate). Lowry C reagent was added to the sample in a volume ratio of 5:1 and stood for 10 minutes. Subsequently, 0.5 portion of the Folin-Ciocalteu reagent was added to the mixture. The reaction was completed after 30 min as evaluated based on the stable highest absorbance at 732 nm. This operating time and λ_{max} were determined in the initial experiment. The protein concentration in the sample was calculated based on the absorbance of the sample and standard (albumin solution in the concentration range of 0.1 to 0.5 mg/mL processed in the same way as the sample).

Determination of alkaline phosphatase enzyme activity

The enzymatic activity of the sample was determined based on the activity of the contained alkaline phosphatase to release free phosphate from Na-β-glycerophosphate (Ren et al., 2015). Two ml of substrate solution at a concentration of 200 mM was taken precisely and mixed with 0.05 mL of enzyme sample in a 5 mL test tube. A blank mixture was also prepared. The volume of these two mixtures was adjusted to 2.2 mL using distilled water. In the third test tube, 0.05 mL of sample and 0.15 mL of water were mixed and referred to as a control mixture. The mixtures were incubated at 38°C for 60 minutes followed by ending the reaction by the addition of 1.8 mL of TCA solution. Thereafter, the control mixture was supplemented with 2.0 mL of substrate solution. Centrifugation at 7500 rpm was applied to these mixtures for 30 minutes and 2.0 mL of supernatant was reacted with 1.6 mL of molybdc acid and 0.4 mL of SnCl₂ for 10 minutes. The concentration of free phosphate anion was determined spectrophotometrically at λ_{max} 630 nm by considering the control and blank absorbance and the concentration was calculated based on the absorbance of samples and standard phosphate solutions prepared using potassium dihydrogen phosphate in the phosphate concentration range of 0.5 to 1 μg/mL. Specific alkaline phosphatase activity (As) is defined as the rate of phosphate release (μg/min) by each unit mass of protein (mg) and calculated using equation 3.

$$As = \frac{[P]}{[protein]t} \quad (3)$$

in which [P] and [protein] are the concentration of released phosphate (μg/ml) and protein (mg/ml), respectively, while t is the reaction time (60 min).

Data Analysis

The rate of germination, protein concentration, and alkaline phosphatase activity of each treatment group were compared with the control group using the student t-test. The statistical analysis was carried out at each germination time using Excel 2016 software at a confidence level of 0.95.

RESULTS AND DISCUSSION

Carcinogenic compound determination is a crucial effort to reduce the incidence and prevalence of cancer, the second-rank disease causing death in the world and the USA (Ahmad et al., 2023; Dattani et al., 2023). The development of fast, cheap, and reliable in vitro carcinogenic substance detection methods is emerging to support efforts to fight cancer. In this research, a fairly simple method for identifying carcinogenic compounds was developed that does not require complex instrumental setup requirements. The technique is based on the influence of carcinogenic substances on the germination of *Raphanus sativus* L. seeds. It has long been known that germination is a sensitive process that is disturbed by small amounts of contaminants in the soil, such as pesticides, which may be carcinogens. As a model carcinogen, a material that is relatively easy to handle, namely hydrazine sulfate, was chosen. This carcinogen is easily soluble in water (National Toxicology Program, 2021), a safe solvent needed in the germination process, for processing *Raphanus sativus* L. seeds. Therefore, only hydrazine sulfate, is the only poison introduced in seed germination. This substance acts as a carcinogen in several mechanisms of action, both directly and through bioactivation as depicted in Figure 1, namely by producing acetohydrazide (Bollard et al., 2005). The next process of Acetohydrazide is the homolytic cleavage of the amide bond to produce acetyl free radicals which can bind to living tissue, including DNA (Qin et al., 2023). This DNA damage can be recovered or some of it will trigger neurodegenerative diseases and can even mutate, causing cancer. (Spencer & Kisby, 2021).

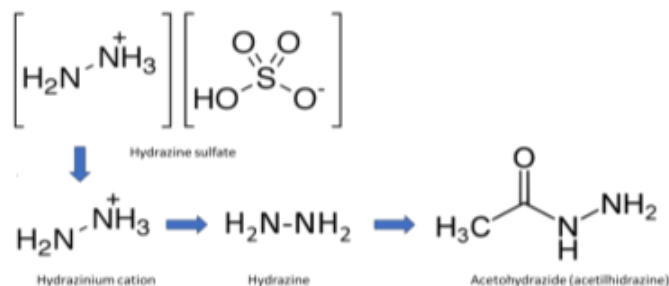


Figure 1. Molecular transformation of hydrazine sulfate to acetohydrazide, a potent source of free radical

Hydrazine sulfate affects germination rate

The concentration-dependent toxic effect of hydrazine sulfate in the range of 1-100 mM delayed *Raphanus sativus* L. seeds germination as shown in Table 1. The minimum concentration of hydrazine sulfate that significantly slowed seeds germination is between 1 and 10 mM. Although only a little information is known regarding the molecular mechanism of hydrazine sulfate toxicity in seeds germination, unlike in animal models and humans (Nguyen et al., 2021), toxicity in various plants and seeds has been recorded for many years (Karnaeva et al., 2022). It may be related to the disruption of ammonia oxidation generating nitrite and nitrate, a key nutrient for plant growth, as seen in lower plants, namely archaea and bacteria (Schatteman et al., 2022). The rate of decline in germination is depicted in Figure 2. Since there was almost no seed germination on media containing 100 mM of hydrazine sulfate, this group of concentration was discarded in the subsequent experiments.

Table 1. Effect of various concentrations of hydrazine sulfate on the germination percentage of *Raphanus sativus* L. seeds

Incubation time (h)	Germination (%)			
	0 mM	1 mM	10 mM	100 mM
24	78 \pm 11	71 \pm 11	49 \pm 16*	3 \pm 2*
48	96 \pm 5	92 \pm 7	78 \pm 19*	7 \pm 4*

Note:

The number is the mean \pm SD of 4 replications.

The sign of * indicates that the germination rate is significantly different compared to the control group (0 mM).

Hydrazine sulfate blocked seeds protein catabolism

In the second stage of germination, approaching the third stage, a small part of the structural protein stored in the seeds is catabolized to produce energy (Müntz et al., 2001), while another part is hydrolyzed into amino acid and converted into a functional protein, for example, enzymes necessary for cell differentiation (He & Yang, 2013; Moubayidin et al., 2010). Therefore, the number of peptide bond somewhat decrease over a certain duration as identified by the Lowry method of protein quantification, resulting in Figure 3. This nitrogenous substances conversion is incomplete due to the toxic activity of hydrazine sulfate at a concentration of 10 mM where the protein content is stable for three days of germination.

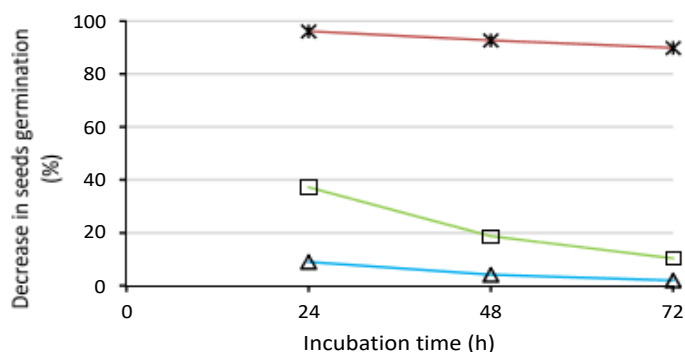


Figure 2. Decrease in seed germination of *Raphanus sativus* L. due to hydrazine sulfate contained in incubation media at concentrations of 1 mM (triangle), 10 mM (square), and 100 mM (star)

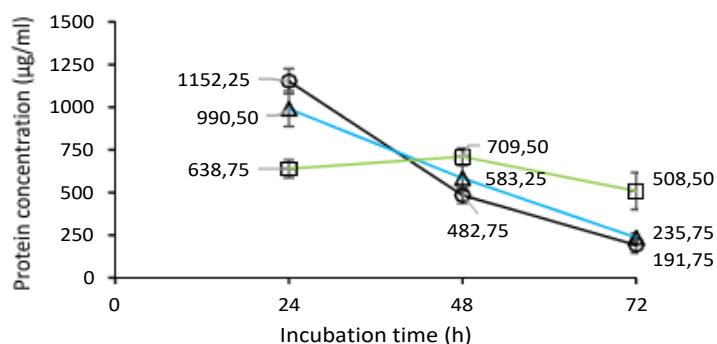


Figure 3. Time course of protein concentration during germination of *Raphanus sativus* L. on media containing hydrazine sulfate at concentrations of 0 mM (control, circle), 1 mM (triangle), and 10 mM (square). The bar lines indicate a standard deviation of 4 replications

Hydrazine sulfate retarded the synthesis of seeds functional protein

Functional proteins are a group of substances showing peptide bonds structurally and exhibiting specific activities, for instance, catalyzing a specific reaction either in vitro or in living tissue. As mentioned above, during seed germination, structural proteins are converted into functional proteins such as some enzymes. The type and level of enzyme depend on the plant and the condition of the seed. These enzymes include protease, xylanase, glucanase, amylase, phosphatase, and so on (Guzmán-Ortiz et al., 2019). Among them, phosphatase has received more attention due to its high increase during germination. This enzyme is needed to release inorganic phosphate from stored organic phosphate, in particular phytic acid. This element is demanded highly during germination for cell division and growth, cell signaling, and metabolic regulation (Prazeres et al., 2004).

In this research, the activity of alkaline phosphatase was determined to assess the expression of this specific protein from DNA. Figure 4 shows that the specific activity of alkaline phosphatase is increased

from day 1 to 3 in the control group, whereas in the treatment group, particularly at 10 mM hydrazine sulfate, a significant decrease appeared.

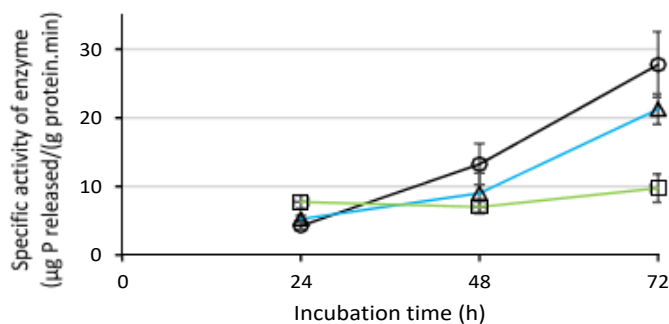


Figure 4. The specific activity of alkaline phosphatase during germination of *Raphanus sativus* L. on media containing hydrazine sulfate at concentrations of 0 mM (control, circle), 1 mM (triangle), 10 mM (square). The bar lines indicate a standard deviation of 4 replications

It can be stated from the analysis of Figures 3 and 4 that in the treatment group of 10 mM hydrazine sulfate, the expression of functional protein was imperfect since the high level of protein was not accompanied by high enzymatic activity, especially after 72 hours of incubation. In Kingdom Plantarum, DNA mutation caused by hydrazine sulfate is evaluated in yeast (*Saccharomyces cerevisiae*) (Government of Canada, 2010). Unfortunately, there is not any information regarding the functional proteins of higher plants which are affected by hydrazine toxicity. In animal models, hydrazine exposure causes down-regulation and up-regulation of several proteins responsible for lipid metabolism, stress response, and thyroid hormone pathways. This includes the up-regulation of transthyretin, thioredoxin peroxidase, and ERp29 (Klenø et al., 2004; Persson, 2002). Regarding the enzyme of interest in this research, alkaline phosphatase of postnatal hamster intestinal tissue increased its activity due to oral administration of hydrazine during gestation (Assessment & Shannon, 2009). The activity enhancement of alkaline phosphatase has been correlated with cancer in animal models (Acharya et al., 2017; Bernhard & Rosenbloom, 1950; Rao et al., 2017). Inversely in breast and colorectal cancer, the activity of alkaline phosphatase decreased (Jiang et al., 2023). Therefore, changes in alkaline phosphatase, whether up- or down-regulated, indicate the possibility of cancerous tissue growth, possibly including in plants, especially during seeds germination.

CONCLUSION

In this research, an early step of the detection method of carcinogenic compounds has been developed. The significant decrease in seed germination rate of *Raphanus sativus* L. by hydrazine sulfate was associated with lower expression of alkaline phosphatase. This alteration has the potential to be a sign of cancer growth and can be adopted in screening for carcinogenic substances.

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