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Review

Evaluation of toxicity and clot lysis activity of thymol.

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Abstract: Thymol (TML) is a highly adaptable compound that has numerous practical uses in fields including medicine, dentistry, veterinary science, food production, and agriculture, among many others. The focus of our study was twofold: firstly, to investigate the toxicity of TML, and secondly, to explore the potential thrombolytic activity of TML. The investigation was carried out to assess the toxicity of TML using the eukaryotic test model Allium cepa (A. cepa). To conduct the experiment, various concentrations of TML were applied to A. cepa for 24, 48, and 72 hours. Distilled water was used as a vehicle while, CuSO4 (0.6 µg/mL) served as the positive control. The length of the roots of the onions was measured in millimeters, and the findings indicated that TML exhibited toxicity in onions in a manner that depended on both the concentration and duration of exposure. At lower concentrations, the longest root length was observed, whereas root growth was hindered with increasing concentrations of TML and exposure time. This inhibition was attributed to the deposition of chemicals and interference with cell division in the root meristematic region of A. cepa. To investigate the thrombolytic ability of TML, an experimental model (in vitro) was used in a laboratory setting. Streptokinase was utilized as a reference or positive control, while water was used as a negative control. The findings of the study revealed that TML exhibited a moderate percentage of clot lysis compared to streptokinase. This effect was dependent on the dosage of TML, and the clot-lytic activity of TML was considerably lower than that of streptokinase.

Keywords: Thymol; eukaryotic test model; root growth inhibition; thrombolytic effect

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1. Introduction

Toxicology is a field of study that deals with the harmful effects of chemical substances on living organisms. Exposure to these substances can happen through various routes such as skin contact, ingestion, or inhalation [4]. Testing for toxicity is important to establish a foundation for regulating substances that may have contact with humans and other living beings, whether deliberately or accidentally. This type of testing is employed to evaluate the safety of a range of products, including cosmetics, pharmaceuticals, food additives, pesticides, chemicals, and other consumer goods [16]. A substance, whether naturally occurring or human-made, can have toxic effects that may cause a range of symptoms, both short-term and long-term in nature [43]. Toxicity testing utilizes different techniques and levels of exposure to the test subject in order to attain a more precise assessment of the potential risks that a particular substance may pose to human health and the surrounding ecosystem [4].

Toxicity testing is crucial in the process of evaluating newly developed drugs before they can be administered to humans. The main goal of conducting toxicity tests is not solely to determine the safety of a test material, but also to detect and explain any possible toxic impacts that it could produce [4]. A variety of tests and organisms are employed to estimate the toxic properties of chemical substances. One such effective test is the Allium cepa (A. cepa) test, which has been shown to have a strong correlation with the outcomes of other eukaryotic test systems and is commonly used to investigate toxic effects [28]. A range of bio-indicator organisms, including A. cepa, are extensively employed to assess the potential risks associated with different types of chemicals [13]. International programs like the Environmental Protection Agency (EPA) and the United Nations Environment Program have established standardized protocols for using bio-indicator organisms in risk assessment. Both the EPA and the World Health Organization acknowledge the usefulness of the data attained from these risk assessments in detecting genotoxicity [9].

Plant models can be used as effective substitutes for animal models in preliminary screening of cytogenotoxicity for different products, due to the ethical concerns regarding the utilize of animal models in toxicity testing. Among the various models employed in toxicity bioassays, the A. cepa test system has been acknowledged as an affordable and sensitive approach to identify genotoxic and cytotoxic hazards associated with environmental chemicals [14,30]. The cytogenotoxic impact observed in the A. cepa test system can provide insight into potential adverse effects in other biological systems, as DNA is a universal component found in all organisms, including humans. This is especially relevant for genotoxicants, such as mutagens, which target DNA as their primary site of action [21,27]. A. cepa is a widely used plant model system, and studies have demonstrated that it is 82% as sensitive as animal models in detecting aberrations in carcinogenicity testing. These findings indicate that mutagenicity and genotoxicity assays conducted using A. cepa can be regarded as comparable to those carried out using mammals [6,31].

When hemostasis fails, a blood clot or thrombus can form in the circulatory system, leading to blockages in blood vessels. In the context of atherothrombotic diseases like acute myocardial or cerebral infarction, this can result in severe consequences and even death during recovery. To address this, thrombolytic agents such as urokinase, anistreplase, alteplase, tissue plasminogen activator (tPA), and streptokinase are commonly used to dissolve blood clots [10]. Although thrombolytic agents are frequently employed to break down blood clots, they still have significant limitations. For example, they may require considerable doses to achieve complete efficacy, have limited fibrin specificity, and increase the risk of bleeding. As a result, Scientists are currently endeavoring to create enhanced versions of these medications using genetic engineering techniques, in order to overcome their limitations [23]. Heparin and aspirin are relatively safe but only provide moderate efficacy in terms of accelerating lysis and preventing reocclusion [3].

Thymol (TML) is a type of phenolic compound that is prominently present in natural sources such as thyme oil and other plants [8]. The compound is a white aromatic crystal present in many essential oils [25]. TML can be extracted from different plants including Coridithymus, Satureja, Lippia, Euphrasia rostkoviana, and Thymus [22]. The compound has been the subject of research due to its potential medicinal benefits, including antioxidant, anti-inflammatory, anesthetic, analgesic, anti-infective, antibacterial, and antifungal effects [33,36]. Studies have shown that TML has anticoagulant effects, which may be linked to its ability to inhibit sodium channels within cells [18]. Recent studies have provided evidence supporting TML's anticancer properties, which are believed to be due to its ability to suppress the growth of cancer cell, trigger cell death, and induce the dissipation of the mitochondrial membrane potential [20]. It is noteworthy that research has shown TML to be effective against the influenza virus and respiratory syncytial virus [26]. TML has not only been investigated for its pharmacological applications, but it has also been sanctioned by the FDA (Food and Drug Administration) in the United States as a safe food additive with negligible toxicity. It has been included in the 'Generally Recognized as Safe' (GRAS) list [12].

The objective of this study was twofold: first, to estimate the potential toxic effects of TML on A. cepa in the presence of copper sulfate-induced toxicity; secondly, to examine whether TML possesses any thrombolytic activity, through an in-vitro method.

2. Materials and methods

1. Collection of Allium cepa, chemical reagents or standards

The toxicity analysis involved using medium-sized onions (A. cepa) that were bought from a local market in Ghonapara, Bangladesh. Analytical-grade copper sulfate pentahydrate ($CuSO_4.5H_2O$) was obtained commercially from Merck, India, while the procurement of streptokinase (SK) was carried out from Dong Kook Pharm. Ltd., which is a Korean-based company. All the other required chemicals and reagents utilized in this investigation were of analytical quality and procured from Merck, India. Distilled water (DW) and other requisite tools were acquired from local markets in Ghonapara, Bangladesh.

2. Preparation of test concentrations

Prior to the study, a literature review was conducted to determine appropriate concentrations of TML to use. Three concentrations of TML were selected, including one below and one above the approved dose. TML and the standard (copper sulfate) are both highly soluble in water. To prepare the highest concentration, solid crystals of copper sulfate and TML were dissolved in water (DW) with manual intermittent shaking for approximately 30 minutes, resulting in a mother solution (MS). The MS was then diluted with DW to achieve the desired concentrations.

3. Study protocols

2.3.1 Allium cepa test

The study utilized A. cepa bulbs that were gathered from a local market. By utilizing a razor blade, the outer scales of the onion bulbs were eliminated and exposed the root primordial through careful scraping. The bulbs were subsequently introduced into glass tubes containing DW and kept in darkness for 24 hours to encourage the growth of roots. Healthy bulbs that had produced rootlets measuring 1 mm in length were chosen, and then transferred to individual glass tubes completely filled with a number of selected concentrations of TML (10, 20, 30, 40, and 50 μg/mL) that had been dissolved in DW. Bulbs that exhibited acceptable root development were transferred to vessels (five for each concentration) holding the specimen/control for durations of 24, 48, and 72 hours. After the culmination of the exposure period, the length of roots were counted and assessed in mm. The hindrance of root expansion was computed to assess the harmful effects of TML, and the IC50 value was calculated for the test sample. Negative and positive controls, consisting of DW and $CuSO_4.5H_2O$ (0.6 μg/mL), respectively, were also used.

2.3.2 Clot lysis test

To conduct the thrombolytic activity test, blood samples were obtained from healthy human volunteers (n = 3) with no history of blood disorders, oral contraceptive use, or ongoing anticoagulant therapy. The blood was collected in sterilized tubes (volume 1.5 mL) and incubated for 45 minutes at 37 °C to allow clot formation. Once the blood had coagulated, the serum was carefully excluded from each tube without disrupting the clot, and the weight of each tube containing the clot was measured. Different concentrations of TML (30, 40, and 50 μg/mL) were introduced into the labeled tubes containing clots, and the positive control tubes were treated with 100 μL of SK, whereas the negative non-thrombolytic control tubes received DW. All tubes were then incubated at a temperature of 37 °C for duration of 90 minutes and assessed for evidence of clot lysis. Once the incubation phase was complete, the liquid was excluded from the tubes, and the tubes were weighed again to determine the disparity in weight before and after the dissolution of the clot. This difference was then quantified as a percentage of clot lysis.

4. Statistical analysis

The study results were expressed as mean ± standard error of mean (SEM) or as a percentage. Statistical analysis of the data was done utilizing analysis of variance (ANOVA) and Tukey post-test with Graph Pad Prism (version 6.0) software. A confidence level of 95% was considered statistically significant with a p-value of less than 0.05.

3. Results

1 Toxic effects of TML on A. cepa

Table 1 displays the mean root length in millimeters of various treatment groups at 24, 48, and 72 hours. The negative control (NC) group demonstrated the highest root growth (RG) at all three time points. The standard $CuSO_4$ treatment decreased RG compared to the NC group at all exposure durations. Interestingly, the group treated with TML at a lower concentration (10 μg/mL) had a high RG profile, but as the concentration of TML enhanced gradually (20-50 μg/mL), the RG profile decreased. This suggests that TML may have a dose-dependent effect on root growth, whereby lower concentrations facilitate growth while higher concentrations impede it (Figure 1).

The A. cepa test revealed a toxic effect that varied depending on the concentration and duration of exposure. The highest level of root growth (RG) suppression was inspected at an exposure time of 72 hours with a concentration of 50 μg/mL, resulting in 86.80% inhibition. Conversely, the lowest RG inhibition of 15.76% was seen with an exposure time of 24 hours at a concentration of 10 μg/mL of TML. The half-minimal inhibitory concentrations (IC50s) were calculated for each exposure time: 34.88 ± 0.03 for 24 hours, 22.64 ± 0.04 for 48 hours, and 12.92 ± 0.03 for 72 hours. These values indicate the concentration of TML needed to achieve 50% inhibition of RG at each time point, with lower values indicating a more potent inhibitory effect. However, TML at all the tested concentrations was found to exert significant ($p< 0.05$) toxic effects on the roots of A. cepa at all exposure times.

Treatments		**Root length (mm)**			**% inhibition of RG**			**IC_{50} (μg/mL)[CI (μg/mL); R^2]**		
		24 h	48 h	72 h	24 h	48 h	72 h	24 h	48 h	72 h
NC		55.50±4.20	79.25±7.59	125.00±6.05	-	-	-	-	-	-
PC		8.75±1.71*	10.00±1.41*	12.00 ± 2.16*	84.23	87.38	90.04	-	-	-
TML (μg/mL)	10	46.75±9.98*	55.75±4.35*	72.75±12.36*	15.76	29.65	41.80	34.88 ± 0.03 [29.92 – 41.07; 0.96]	22.64 ± 0.04 [15.59 – 24.75; 0.96]	12.92 ± 0.03 [10.76 – 15.50; 0.98]
	20	30.25±7.27*	41.50±8.18*	43.25±5.38*	45.49	47.63	65.40			
	30	28.50±9.29*	31.00±8.75*	32.50±8.66*	48.65	60.88	74.00			
	40	13.50±3.11*	15.25±2.63*	17.50±2.89*	75.67	80.76	86.00			
	50	12.50±2.64*	14.00±3.26*	16.50±3.41*	77.48	82.33	86.80			

Values are mean ± standard error of the mean (SEM) (n = 4); *p<0.05 when compared to the vehilce (NC) group; ANOVA followed by Tukey post hoc test, considering $p< 0.05$ with a confidence level of 95%; Negative values are omitted in the table; TML: Thymol; NC: negative control; PC: positive control ($CuSO_4 .5H_2O$); RG: root growth; IC_{50}: half maximal inhibitory concentration; CI: confidence of interval; R^2: coefficient of determination at 95% confidence intervals

Table 1. Toxic effects of TML and the controls on Allium cepa root meristems at different exposure times.

The percentage of adaptation towards toxicity of TML has been presented in figure 1. The findings demonstrated that the percentage of adaptation gradually enhanced toward $CuSO_4$-induced toxicity with increasing exposure time. The result predicts that the highest percentage of adaptation against TML toxicity is 49.37% at 72 hours at the concentration of TML (30 μg/mL) inspect of 24 hours, but depletion was observed at 48

hours. The result also indicates that the lowest percentage of adaptation toward the toxicity is 3.92% at 20 μg/ml at 48 hours for 24 hours.

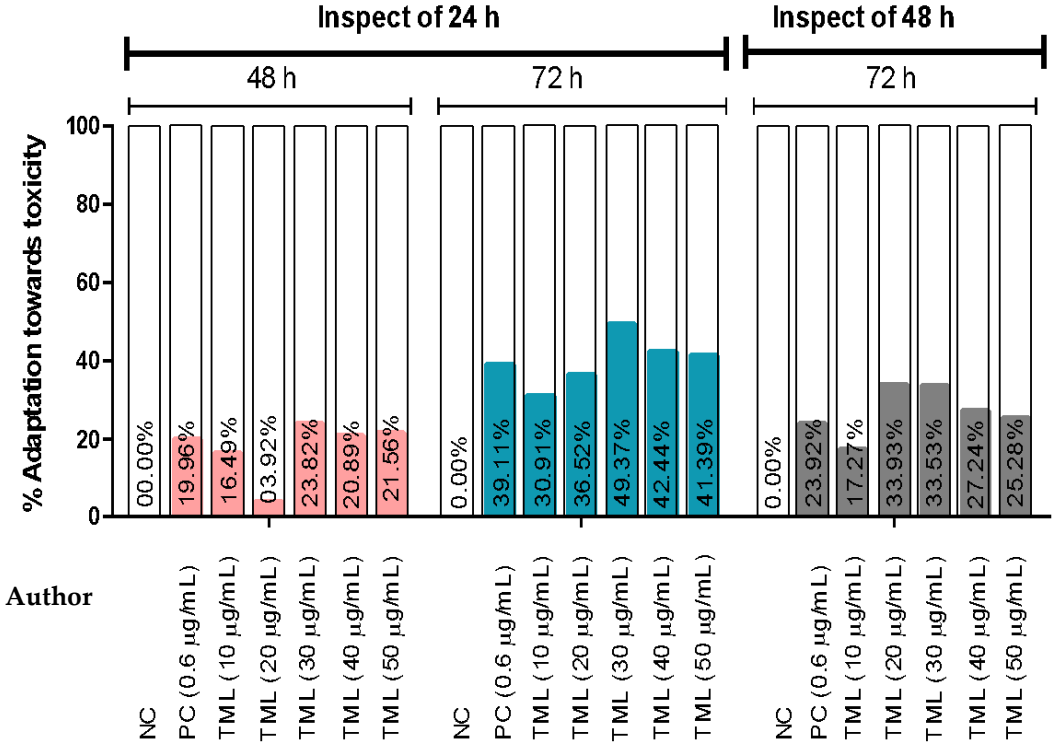

Figure 1. Adaption towards the toxic effects of TML (TML) and controls on Allium cepa inspect of 24 and 48 h of exposure time [Values are percentage decrease in toxic response in the same group of treatment, inspect of 24 and 48 h of exposure time. Negative values are omitted in the graph and are represented as 0.00%. NC: distilled water; PC: positive control ($CuSO_4.5H_2O$)]

2. Clot lysis activity

When SK (100 μL), a positive control, was incorporated into the clots and incubated at a temperature of 37°C for a duration of 90 minutes, 76.61% clot lysis was observed. In contrast, when clots were treated with DW (negative control), only a minimal 3.14% clot lysis was observed. There was a notable difference in clot lysis percentage between the positive and negative controls. TML demonstrated clot lysis capacity in a manner that was dependent on its concentration. The highest degree of clot lysis (28.53 ± 1.95) % was estimated at a concentration of 55 μg/mL, while the lowest degree of clot lysis (19.72 ± 2.87) % was estimated at a concentration of 10 μg/mL. Upon comparing the clot lysis ability of the test groups with the SK group, it was evident that both TML and the SK group exhibited substantial clot lysis effects in contrast to the NC group. The half-minimal inhibitory concentration (IC50) was 79.90 ± 0.14. However, TML at all the tested concentrations was found to exert significant ($p< 0.05$) clot lysis effects (Table 2).

Treatments	Concentration	%clot lysis	IC_{50} (μg/mL) [CI (μg/mL); R^2]
NC	-	3.14±0.16	-
SK	30,000 I.U	76.61 ± 2.98*	-
TML (μg/mL)	10	19.72 ± 2.87*	79.90 ± 0.14 [18.04 – 270.90; 0.87]
	30	22.26 ± 2.33*	
	55	28.53 ± 1.95*	

Values are mean ± SEM (n = 3); *p<0.05 when compared to the vehilce (NC) group; ANOVA followed by Tukey post hoc test, considering p<0.05 with a confidence level of 95%; NC (100 μL): DW(vehicle); SK (100 μL (30,000 I.U.)): SK (positive control)

4. Discussion

The A. cepa test is a commonly used method to investigate the pro-oxidative or toxicogenetic characteristics of various substances, such as isolated compounds, crude extracts, heavy metals, other biochemicals, as well as anti-cancer agents and antioxidants [34,35]. The accumulation of toxic substances in the roots of A. cepa can lead to the inhibition of root growth and chromosomal aberrations, such as micronuclei, chromosomal bridges, C-mitosis, and chromosomal tack. The accumulation of toxic substances at the site of the meristematic cells in the root tip may be the reason for the significant disruption of microtubule arrangements in this particular eukaryotic testing system [15,38,42]. To better understand the effects of any substance, it is important to investigate various chromosome disturbances that can occur during cell division, in addition to breaks. The A. cepa assay is a well-known and frequently used method for this purpose. It has been suggested as a standard test material due to its ease of use, sensitivity, and the fact that it does not require an expensive or complex laboratory setup [2].

In the A. cepa test model, the root growth (RG) profile is a significant parameter that can help to understand the toxic effects of a material [14]. In this model, the exposure of a test substance can lead to a decrease in root length, which indicates the toxic effects of the material [1]. In this model, a substance such as Copper (Cu) is considered a standard due to its absorption by plants from the environment and subsequent accumulation generally in the roots. This is because of the movement of Cu within the plant is physiologically regulated [41,17]. Even though Cu is a necessary metal ion, excessive amounts of its absorption can lead to extreme impairments to the growth and development of plants in this model [11]. In this model, Cu was found to accumulate in the roots of A. cepa and remarkably hinder RG at an early stage. Distinct effects were observed between low and high Cu concentrations [32]. In our investigation, we found that exposure to $CuSO_4$ at 0.6 μg/mL led to suppression of RG across all exposure times, with the % inhibition of RG increasing over time. TML exhibited a concentration-dependent effect, with higher RG and lower % inhibition of RG at low concentrations, and lower RG and higher % inhibition of RG at high concentrations. This suggests that TML has cytotoxic properties that lead to inhibition of RG in A. cepa, which is likely related to impaired cell elongation during

differentiation [15], the inhibition of protein synthesis, and the reduction of apical meristematic activity [28,38].

When blood clots form within blood vessels, they can suppress the flow of blood throughout the body's circulatory system, which can result in conditions such as hypertension, stroke, anoxia, and other related conditions [5,19,24]. If it occurs in the brain, it can cause damage to the nerve cells, which can eventually result in neurological degeneration [37]. Thrombolytic medications are primarily administered to manage patients with thrombosis [7]. Based on our investigation, we discovered that TML has a moderate potential for clot lysis. Our findings indicated that a high concentration of TML resulted in a clot lysis of 28.53 %, which was significantly different from the NC group.

5. Conclusion

TML exhibited a toxic effect in the A. cepa test that was dependent on both concentration and exposure time. As the concentration and duration of exposure increased, the percentage of root growth was reduced because of chromosomal abnormalities in the root meristems of A. cepa. The group treated with $CuSO_4$ showed the highest percentage of inhibition of root growth (IRG) after 72 hours of exposure. TML also had a significant inhibitory effect on root growth, particularly at higher concentrations and longer exposure times. Furthermore, the protective effects of TML on root growth were observed to increase with time, up to 72 hours, compared to 24 hours, but a decrease in the percentage of root growth was noticed after 72 hours compared to 48 hours. Our study indicates that TML exhibited toxicity in a concentration-dependent manner in this test system of eukaryotes. Therefore, it is crucial to consider the traditional and industrial uses of TML with greater caution. Our in vitro study on clot lysis showed that TML has a moderate thrombolytic effect and the response is dose dependent. The thrombolytic activity was greater with the higher dose (55 μg/mL) than with the lower dose (10 μg/mL). Therefore, it can be concluded that TML may serve as a promising natural source of thrombolytic agents.

Conflict of Interest: No conflict of interest to disclose.

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References

1. Adeyemo, O. A., & Farinmade, A. E. (2013). Genotoxic and cytotoxic effects of food flavor enhancer, monosodium glutamate (MSG) using Allium cepa assay. African Journal of Biotechnology, 12(13).
2. Ahmed, T., Torequl Islam, M., & Rouf, R. (2020). Toxic/protective effects of some antioxidants on the copper sulfate induced toxicity in Allium cepa. Journal of Pharmaceutical and Applied Chemistry, 6(2), 43-48.
3. Anwar, M. S., Khan, I. N., Barua, S., Kamal, A. M., Hosen, S. Z., & Kawsar, M. H. (2011). Assessment of thrombolytic & cytotoxic activity of herbal preparations originated from botanical source of Bangladesh. Journal of Applied Pharmaceutical Science, (Issue), 77-80.
4. Arome, D., & Chinedu, E. (2013). The importance of toxicity testing. Journal of Pharmaceutical and BioSciences, 4, 146-148.

5. Bentzon, J. F., Otsuka, F., Virmani, R., & Falk, E. (2014). Mechanisms of plaque formation and rupture. Circulation research, 114(12), 1852-1866.
6. Bernardes, P. M., Andrade-Vieira, L. F., Aragão, F. B., Ferreira, A., & da Silva Ferreira, M. F. (2015). Toxicity of difenoconazole and tebuconazole in Allium cepa. Water, Air, & Soil Pollution, 226(7), 1-11.
7. Biswas, K., Hossain, S., Alam, S., Sultana, T., Khan, F., & Dutta, S. (2018). In-vitro Assessment of cholinesterase inhibitory and thrombolytic activity of six available citrus fruits in Bangladesh: Relevant for Treating Neurodegenerative Disorder. European Journal of Medicinal Plants, 5, 1-1.
8. Braga, P. C., Dal Sasso, M., Culici, M., Bianchi, T., Bordoni, L., & Marabini, L. (2006). Anti-inflammatory activity of TML: inhibitory effect on the release of human neutrophil elastase. Pharmacology, 77(3), 130-136.
9. Çavuşoğlu, D., Çavuşoğlu, K., & Tabur, S. (2006). THE EFFECTS 0F BLACK CUMIN (NIGELLA SATIVA L.) SEED EXTRACT ON THE SEED GERMINATION, SEEDLING GROWTH, MITOTIC ACTIVITY AND CHROMOSOMAL ABBERATIONS OF ALLIUM CEPA L. UNDER SALINE CONDITION.
10. Collen, D. (1990). Coronary thrombolysis: streptokinase or recombinant tissue-type plasminogen activator?. Annals of internal medicine, 112(7), 529-538.
11. Ducic, T., & Polle, A. (2005). Transport and detoxification of manganese and copper in plants. Brazilian Journal of Plant Physiology, 17, 103-112.
12. Escobar, A., Perez, M., Romanelli, G., & Blustein, G. (2020). TML bioactivity: A review focusing on practical applications. Arabian Journal of Chemistry, 13(12), 9243-9269.
13. Firbas, P., & Amon, T. (2014). Chromosome damage studies in the onion plant Allium cepa L. Caryologia, 67(1), 25-35.
14. Fiskesjö, G. (1985). The Allium test as a standard in environmental monitoring. Hereditas, 102(1), 99-112.
15. Fusconi, A., Repetto, O., Bona, E., Massa, N., Gallo, C., Dumas-Gaudot, E., & Berta, G. (2006). Effects of cadmium on meristem activity and nucleus ploidy in roots of Pisum sativum L. cv. Frisson seedlings. Environmental and Experimental Botany, 58(1-3), 253-260.
16. Gallagher, M. E. (2002). Toxicity testing requirements, methods and proposed alternatives. Environs: Envtl. L. & Pol'y J., 26, 253.
17. Geremias, R., Fattorini, D., Fávere, V. T., & Pedrosa, R. C. (2010). Bioaccumulation and toxic effects of copper in common onion Allium cepa L. Chemistry and Ecology, 26(1), 19-26.
18. Haeseler, G., Maue, D., Grosskreutz, J., Bufler, J., Nentwig, B., Piepenbrock, S., & Leuwer, M. (2002). Voltage-dependent block of neuronal and skeletal muscle sodium channels by TML and menthol. European journal of anaesthesiology, 19(8), 571-579.
19. Holy, E. W., Besler, C., Reiner, M. F., Camici, G. G., Manz, J., Beer, J. H., & Tanner, F. C. (2014). High-density lipoprotein from patients with coronary heart disease loses anti-thrombotic effects on endothelial cells: impact on arterial thrombus formation. Thrombosis and haemostasis, 112(11), 1024-1035.
20. Kang, S. H., Kim, Y. S., Kim, E. K., Hwang, J. W., Jeong, J. H., Dong, X., & Park, P. J. (2016). Anticancer effect of TML on AGS human gastric carcinoma cells. Journal of microbiology and biotechnology, 26(1), 28-37.
21. Leme, D. M., & Marin-Morales, M. A. (2009). Allium cepa test in environmental monitoring: a review on its application. Mutation research/reviews in mutation research, 682(1), 71-81.
22. Marchese, A., Orhan, I. E., Daglia, M., Barbieri, R., Di Lorenzo, A., Nabavi, S. F., & Nabavi, S. M. (2016). Antibacterial and antifungal activities of TML: A brief review of the literature. Food chemistry, 210, 402-414.
23. Marder, V. J. (1993). Recombinant streptokinase: Opportunity for an improved agent. Blood Coagulation & Fibrinolysis, 4(6), 1039-1040.
24. Matsuura, Y., Yamashita, A., Iwakiri, T., Sugita, C., Okuyama, N., Kitamura, K., & Asada, Y. (2015). Vascular wall hypoxia promotes arterial thrombus formation via augmentation of vascular thrombogenicity. Thrombosis and haemostasis, 114(07), 158-172.
25. Nabavi, S. M., Marchese, A., Izadi, M., Curti, V., Daglia, M., & Nabavi, S. F. (2015). Plants belonging to the genus Thymus as antibacterial agents: From farm to pharmacy. Food chemistry, 173, 339-347.
26. Najafloo, R., Behyari, M., Imani, R., & Nour, S. (2020). A mini-review of TML incorporated materials: Applications in antibacterial wound dressing. Journal of Drug Delivery Science and Technology, 60, 101904.
27. Nawalage, S. K., & Pathiratne, A. (2020). Application of cytogenetic model Allium cepa for screening potential cytogenotoxicity of herbal-based hair dyes. Journal of Environmental Science and Health, Part A, 55(11), 1366-1372.
28. Özkara, A., Akyıl, D., Eren, Y., & Erdoğmuş, S. F. (2015). Potential cytotoxic effect of Anilofos by using Allium cepa assay. Cytotechnology, 67(5), 783-791.
29. Öztürk, G., Çavuşoğlu, K., & Yalçın, E. (2020). Dose–response analysis of potassium bromate–induced toxicity in Allium cepa L. meristematic cells. Environmental Science and Pollution Research, 27(34), 43312-43321.
30. Pathiratne, A., Hemachandra, C. K., & De Silva, N. (2015). Efficacy of Allium cepa test system for screening cytotoxicity and genotoxicity of industrial effluents originated from different industrial activities. Environmental Monitoring and Assessment, 187(12), 1-12.
31. Pérez, D. J., Lukaszewicz, G., Menone, M. L., & Camadro, E. L. (2011). Sensitivity of Bidens laevis L. to mutagenic compounds. Use of chromosomal aberrations as biomarkers of genotoxicity. Environmental Pollution, 159(1), 281-286.

32. Qin, R., Wang, C., Chen, D., Björn, L. O., & Li, S. (2015). Copper-induced root growth inhibition of Allium cepa var. agrogarum L. involves disturbances in cell division and DNA damage. Environmental toxicology and chemistry, 34(5), 1045-1055.
33. Riella, K. R., Marinho, R. R., Santos, J. S., Pereira-Filho, R. N., Cardoso, J. C., Albuquerque-Junior, R. L. C., & Thomazzi, S. M. (2012). Anti-inflammatory and cicatrizing activities of TML, a monoterpene of the essential oil from Lippia gracilis, in rodents. Journal of Ethnopharmacology, 143(2), 656-663.
34. Saleheen, D., Ali, S. A., & Yasinzai, M. M. (2004). Antileishmanial activity of aqueous onion extract in vitro. Fitoterapia, 75(1), 9-13.
35. Santas, J., Carbo, R., Gordon, M. H., & Almajano, M. P. (2008). Comparison of the antioxidant activity of two Spanish onion varieties. Food Chemistry, 107(3), 1210-1216.
36. Schilcher, H. (1985). Effects and side-effects of essential oils. In Essential oils and aromatic plants (pp. 217-231). Springer, Dordrecht.
37. Schuhmann, M. K., Kraft, P., Stoll, G., Lorenz, K., Meuth, S. G., Wiendl, H., & Kleinschnitz, C. (2015). CD28 superagonist-mediated boost of regulatory T cells increases thrombo-inflammation and ischemic neurodegeneration during the acute phase of experimental stroke. Journal of Cerebral Blood Flow & Metabolism, 35(1), 6-10.
38. Seth, C. S., Chaturvedi, P. K., & Misra, V. (2007). Toxic effect of arsenate and cadmium alone and in combination on giant duckweed (Spirodela polyrrhiza L.) in response to its accumulation. Environmental Toxicology: An International Journal, 22(6), 539-549.
39. Seth, C. S., Chaturvedi, P. K., & Misra, V. (2007). Toxic effect of arsenate and cadmium alone and in combination on giant duckweed (Spirodela polyrrhiza L.) in response to its accumulation.
40. Environmental Toxicology: An International Journal, 22(6), 539-549.
41. Song, J., Zhao, F. J., Luo, Y. M., McGrath, S. P., & Zhang, H. (2004). Copper uptake by Elsholtzia splendens and Silene vulgaris and assessment of copper phytoavailability in contaminated soils. Environmental Pollution, 128(3), 307-315.
42. Webster, P. L., & MacLeod, R. D. (1996). The root apical meristem and its margin. Plant Roots. The Hidden Half, second ed. Marcel Dekker, New York, 51-76.
43. Zurlo, J., Rudacille, D., & Goldberg, A. M. (1994). Animals and alternatives in testing: history, science, and ethics (p. 86). New York: Mary Ann Liebert**.**