Activity of Ethanol Extract of Centella asiatica (Linn.) Urban in Inhibiting the Progression of Buccal Mucosal Dysplasia Lesions in Wistar Rats

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Abstract

Oral epithelial dysplasia (OED) is the most important stage of change and is called a reversible premalignant lesion. Pegagan (Centella asiatica (Linn.) Urban) is one of the herbal plants widely utilized and has the potential cancer chemoprevention agent. This study aimed to determine the effect of Centella asiatica ethanol extract in inhibiting the progression of buccal mucosal dysplasia lesions in Wistar rats histologically with HE staining.

This study is a laboratory experiment with a post-test-only control group design. A total of 28 male Wistar rats were divided into four groups: the control group without treatment and the treatment group were given different doses of Centella asiatica extract (375 mg/kgBW, 750 mg/kgBW, 1500 mg/kgBW) for 61 days. On day 29, all groups of rats had their buccal mucosa induced with 0.5% DMBA three times a week for four weeks. On day 61, all rats were sacrificed, and buccal mucosa samples were examined histopathologically with HE staining. The degree of dysplasia was assessed by scoring based on the WHO 2017 classification. The data were analyzed using the Kruskal-Wallis test and Mann-Whitney test.

The results showed that the average score of the highest degree of dysplasia in the control group (1.63 ± 0.51), then sequentially followed by the treatment group doses of 375 mg/kgBW, 750 mg/kgBW and 1500 mg/kgBW. Statistical tests showed significant differences in all groups ($p < 0.05$). There was a significant difference between the 750 mg/kgBW group and the control group ($p<0.05$) and the 1500 mg/kgBW group with the control group ($p<0.05$).

The administration of high doses of Centella asiatica ethanol extract, namely 750 mg/kgBW and 1500 mg/kgBW, effectively inhibited the progression of buccal mucosal dysplasia lesions in Wistar rats induced with DMBA. In other words, Centella asiatica has the potential as a chemoprevention agent.

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Introduction

Oral cancer is part of head and neck cancer. It is a malignancy in the lip, oral cavity, and pharynx.[1] Oral cancer occurs in nearly 400,000 new patients worldwide each year. This disease is calculated to be the sixth most frequent malignancy.[2] The prevalence of oral cancer in Indonesia is 3-4% of all cancers that occur today. Carcinogenesis is the process of normal cells being transformed into cancer cells, occurring gradually, caused by genetic mutations of normal cells, which cause uncontrolled cell division due to disrupting the balance between cell proliferation and death. Carcinogenesis occurs in three stages, namely initiation, promotion, and progression.[3-5]

Histologically, the progression of oral carcinogenesis begins with the change of hyperplasia cells into dysplasia cells, starting with mild, moderate, and severe dysplasia to become cancer cells that invade and metastasize.[6] Oral epithelial dysplasia (OED) is the most crucial stage of change and is called a reversible

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premalignant lesion.[7] Several studies state that cancer risk prevention can be done by avoiding biological, chemical, and physical sources of cancer and familiarizing the consumption of antioxidant-rich foods and consumption of chemoprevention agents. Cancer chemoprevention is the use of natural, synthetic, or biological chemicals or agents to reverse, suppress, or prevent carcinogenesis, the progressivity of carcinogens to invasive cancer, or the progressivity of tissues at risk of developing invasive cancer.[8-10]

Indonesia is one of the countries rich in various types of plants that can be utilized as anti-cancer drugs. Indonesia, China, Arabia, Japan, and India are countries that have known medicinal plants since long ago. *Centella asiatica* (CA) or pegagan or horse foot leaves is one of the traditional plants that has been widely used in the treatment of various diseases.[11] There are seven main groups of compounds in pegagan: saponins, pentacyclic triterpenoids, sterols, sesquiterpenes, eugenol derivatives, caffeoylquinic acid, and flavonoids. Gotu kola (*Centella asiatica* (Linn.) Urban) is a widely utilized herbal plant and has the potential cancer chemoprevention agent.[12] Therefore, this study aimed to determine the effect of *Centella asiatica* ethanol extract in inhibiting the progression of buccal mucosal dysplasia lesions in Wistar rats histologically with HE staining.

Materials and methods

This study is a laboratory experiment with a post-test-only control group design. The extraction of *Centella asiatica* (Linn.) Urban was carried out by maceration by soaking 1 kg of *Centella asiatica* dry powder in 70% ethanol solvent as much as 15 liters. The powder was soaked in a closed vessel for 6 hours at room temperature, allowed to stand for 18 hours while stirring occasionally, and soaked for 24 hours to obtain the content of *Centella asiatica* (Linn.) Urban. Separate the macerate filtered with filter paper. The filtrate resulting from maceration is then mixed, then evaporated using a waterbath while stirring until a thick extract is obtained. The result is called *Centella asiatica* (Linn.) Urban ethanol extract and stored in plastic pots. A total of 28 male Wistar rats were divided into four groups: the treatment group were given different doses of *Centella asiatica* extract (Group A: 375 mg/kgBW; Group B: 750 mg/kgBW; Group C: 1500 mg/kgBW) for 61 days and Group D: control without treatment. On day 29, all groups of rats in both treatment and control groups were anesthetized with ketamine hydrochloride anesthetic intramuscularly at a dose of 45 mg/kgBW on the buccal mucosa. The buccal mucosa of each rat in the treatment and control groups was then scraped using a 27G syringe containing 100 µl DMBA 0.5% using corn oil as a solvent along 1 cm for three times a week (every Monday, Wednesday, and Friday) for 4 weeks. On day 61, all rats were sacrificed, and buccal mucosa samples were examined histopathologically with HE staining. The degree of dysplasia was assessed by scoring based on the WHO 2017 classification. The data were analyzed using the Kruskal-Wallis test and Mann-Whitney test.

Results

The results showed a histological picture of dysplasia with HE staining based on the WHO 2017 classification. In groups A and D, there were architectural changes in the form of premature keratinization in single cells (dyskeratosis). In contrast, groups B and C showed different changes in the form of irregular epithelial stratification and loss of polarity of basal cells.

In addition, the results of cellular changes in the four groups showed a similar picture in the form of anisonucleosis, nuclear pleomorphism, anisocytosis, cellular pleomorphism, increased nuclear-cytoplasmic ratio, and hyperchromasia. However, groups A and D also showed an increase in the number and size of nucleoli (Figure 2).

Group	Dysplasia Score (Average ± SD)	All Group Analysis (p-value)	Between Group Analysis (p-value)			
			Group A	Group B	Group C	Group D
A	1,38 ± 0,51	0,032[a]	-	-	-	-
B	1,13 ± 0,35		0,264	-	-	-
C	1,00 ± 0,00		0,063	0,317	-	-
D	1,63 ± 0,51		0,333	0,046[b]	0,009[b]	-

Table 1. Differences Dysplasia Score in All Groups.
[a]Kruskal-Wallis statistical test; [b]Mann-whitney statistical test; significance level p<0.05.

The average score of the highest degree of dysplasia can be seen in table 1. In the table 1, control group (1.63 ± 0.51) was then sequentially followed by the treatment group doses of 375

mg/kgBW, 750 mg/kgBW, and 1500 mg/kgBW. Statistical tests showed significant differences in all groups ($p < 0.05$). There was a significant difference between the 750 mg/kgBW group and the control group ($p<0.05$) and the 1500 mg/kgBW group with the control group ($p<0.05$).

Discussion

Based on the results of this study, in the histological picture of the control group and the lowest dose, there is early keratinization in single cells (dyskeratosis) which is early keratinization that occurs in individual cells or groups of cells in different epithelial strata, before reaching the surface. These cells become separated from adjacent cells. These dyskeratotic cells are large and round with deeply eosinophilic cytoplasm and hyperchromatic nuclei. Benign keratinized pearls are surrounded by cells that are not dysplastic; for example, - dyskeratosis. The cells are arranged concentrically when there is a lack of cohesion between epithelial cells due to malignant changes. Since the fate of squamous cells is to form keratin, these cells lay down keratin concentrically and then appear as keratin pearls known as malignant keratin pearls. Keratin pearls are circular accumulations of keratin made by malignant squamous cells and present in concentric layers between squamous epithelium. These different keratin formation patterns depend on the triggering stimulus's amount and nature. In frictional keratosis or mild leukoplakia, if the underlying stimulus is removed, the mucosal changes will return to normal, whereas in squamous cell carcinoma, there is premature keratinization of the cells before they undergo complete differentiation.[13] Whereas the group with higher doses showed irregular epithelial stratification and loss of basal cell polarity, the four groups also experienced similar cellular changes, indicating that there was abnormal cell cycle activity both in each phase and in cells that experienced dysplasia. Dysplasia (dys = abnormal/bad; plasia = growth) is defined as 'A precancerous lesion of stratified squamous epithelium characterized by cellular atypia and loss of normal maturation and stratification shorter than carcinoma in situ'.[7] However, these architectural and cellular changes do not indicate the severity of the cell dysplasia but only that there is dysplasia in the cell.

Based on the comparison of dysplasia scores, the group with *Centella asiatica* ethanol extract was more effective in suppressing the development of precancerous lesions, namely dysplasia, compared to the control group. *Centella asiatica* is one of the traditional plants that has been widely used to treat various diseases.[11] There are seven main groups of compounds in gotu kola: saponins, pentacyclic triterpenoids, sterols, sesquiterpenes, eugenol derivatives, caffeoylquinic acid and flavonoids. Pentacyclic triterpenoids are the highest content, also known as centelloids, where the largest content is saponins, asiaticoside, madecasocide, aglycone, asiatic acid, and madecasic acid.[12] Oral administration of *Centella asiatica* ethanol extract doses of 50 mg / kgBW and 100 mg / kgBW can increase the immune system as indicated by an increase in IgG levels in the blood serum of mice induced by the BCG vaccine.[14] Further research examining the benefits of *Centella asiatica* molecularly and using larger doses was conducted by Mustika et al. in 2021, who examined the effect of peroral administration of *Centella asiatica* ethanol extract to see an increase in apoptosis of alveolar macrophage cells in Mycobacterium tuberculosis-induced mouse models. *Centella asiatica* ethanol extract was divided into 3 doses, namely 375 mg / kgBW, 750 mg / kgBW, and 1500 mg / kgBW, the results of the three doses were significantly different from the control group in increasing apoptosis of rat alveolar macrophage cells, characterized by increased expression of Caspase 8 and Bax, and decreased expression of Bcl-2 which is a biomarker of cell apoptosis, but there was no significant difference between groups given *Centella asiatica* ethanol extract. This study concluded that the best dose of *Centella asiatica* ethanol extract was 750 mg/kgBW (Mustika et al., 2021).[15]

The mechanism of *Centella asiatica* is based on its secondary metabolite content. It has been reported to have several types, such as triterpenoids, volatile and fatty acids, alkaloids, glycosides, flavonoids and others (vitamins B, C, and some amino acids). Triterpenoids include asiaticoside, centelloside, madecossoside, thankuniside, isothankunic acid, centellose acid, asiatic acid, centellic acid, madecassic acid, brahmoside, braminoside, and brahmic acid. The content of asiaticoside and madecossoside is the most in the leaves and a little in the roots.[11,16,17] Flavanoids (3-glucosylquercetin, 3-

glucosylkaemferol, and 7-glucosylkaemferol) can be isolated from plant leaves. *Centella asiatica* has been reported to contain tannins, sugars, inorganic acids and resins, glycine, aspartic acid, glutamic acid, α-alanine, and phenylalanine, while other contents, namely chloride, sulfate, phosphate, iron, calcium, magnesium, sodium, and potassium. The leaves are rich in vitamins such as vitamins B, C, and G.[17] According to Halliwell and Gutteridge (1999) cit. Wahyu et al. (2005), the mechanism of flavonoids acting as antioxidants can be done in a way, first, by suppressing the formation of free radicals or ROS by inhibiting enzymes, metal ion attachment (metal ion chelating) involved in the production of free radicals, then second, by reducing free radicals (free radical scavenger). Flavonoids inhibit enzymes responsible for producing superoxide anion radicals, such as xanthine oxidase and protein kinase. Flavonoids also show inhibition of cyclooxygenase, lipoxygenase, microsomal monooxygenase, glutathione S-transferase, succinct oxygenase, and NADH oxidase, which are all involved in the formation of Reactive Oxygen Species (ROS). ROS are superoxide radicals (O2*-) that can stimulate oxidation or the formation of compounds that can interfere with cell integrity because they can react with cell components both structural and functional components, just like cells that experience dysplasia.[18-20]

Conclusions

According to this study, it can be concluded that Centella asiatica ethanol extract can inhibit the progression of precancerous lesions (dysplasia) to malignancy, especially Centella asiatica ethanol extract doses of 750 mg/kgBW and 1500 mg/kgBW. In other words, the higher the dose given, the better the effect will be and also Centella asiatica can be used as a candidate herbal plant as a chemopreventive agent for oral cancer.

Declaration of Interest

The authors report no conflict of interest.

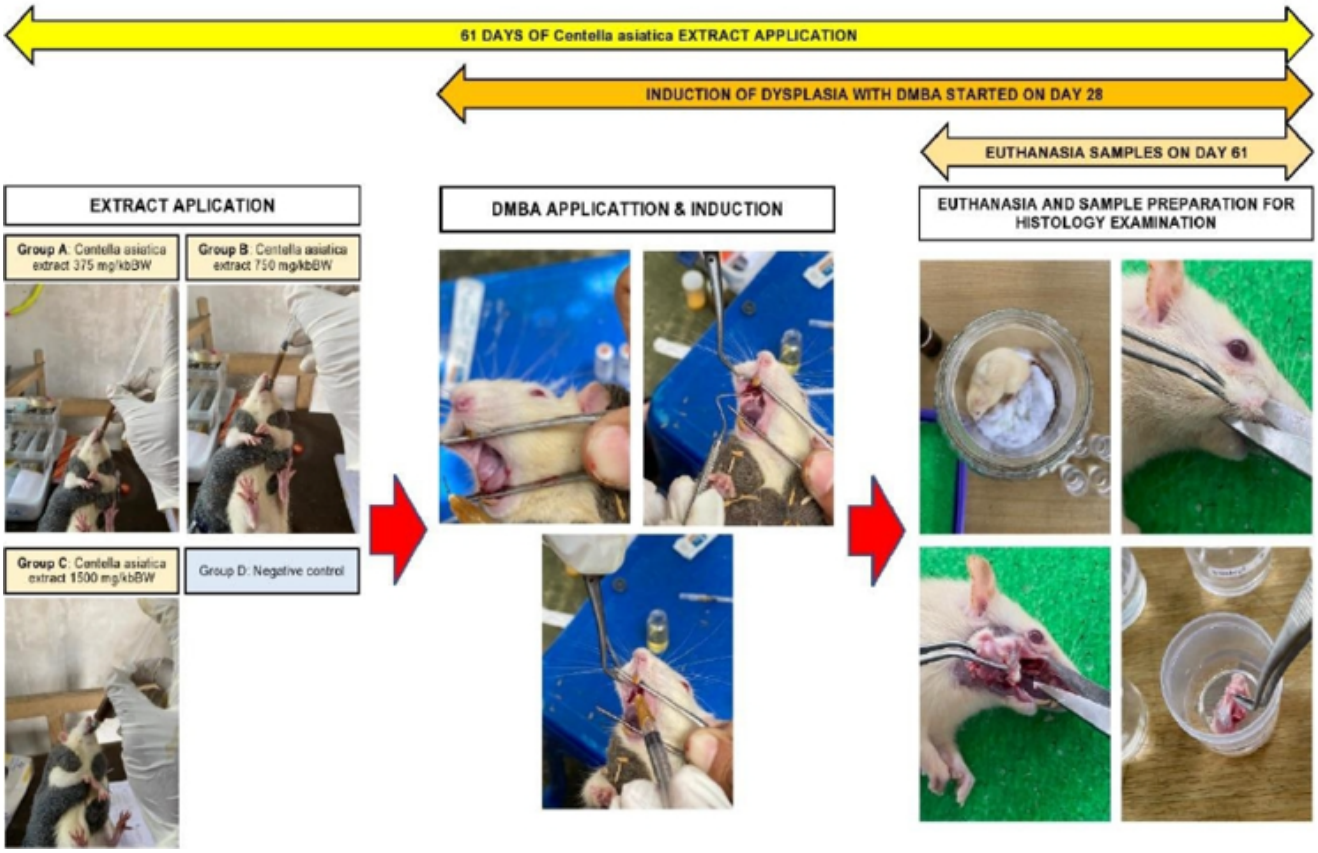

Figure 1. The procedure of treatment and examination of rat samples in the study.

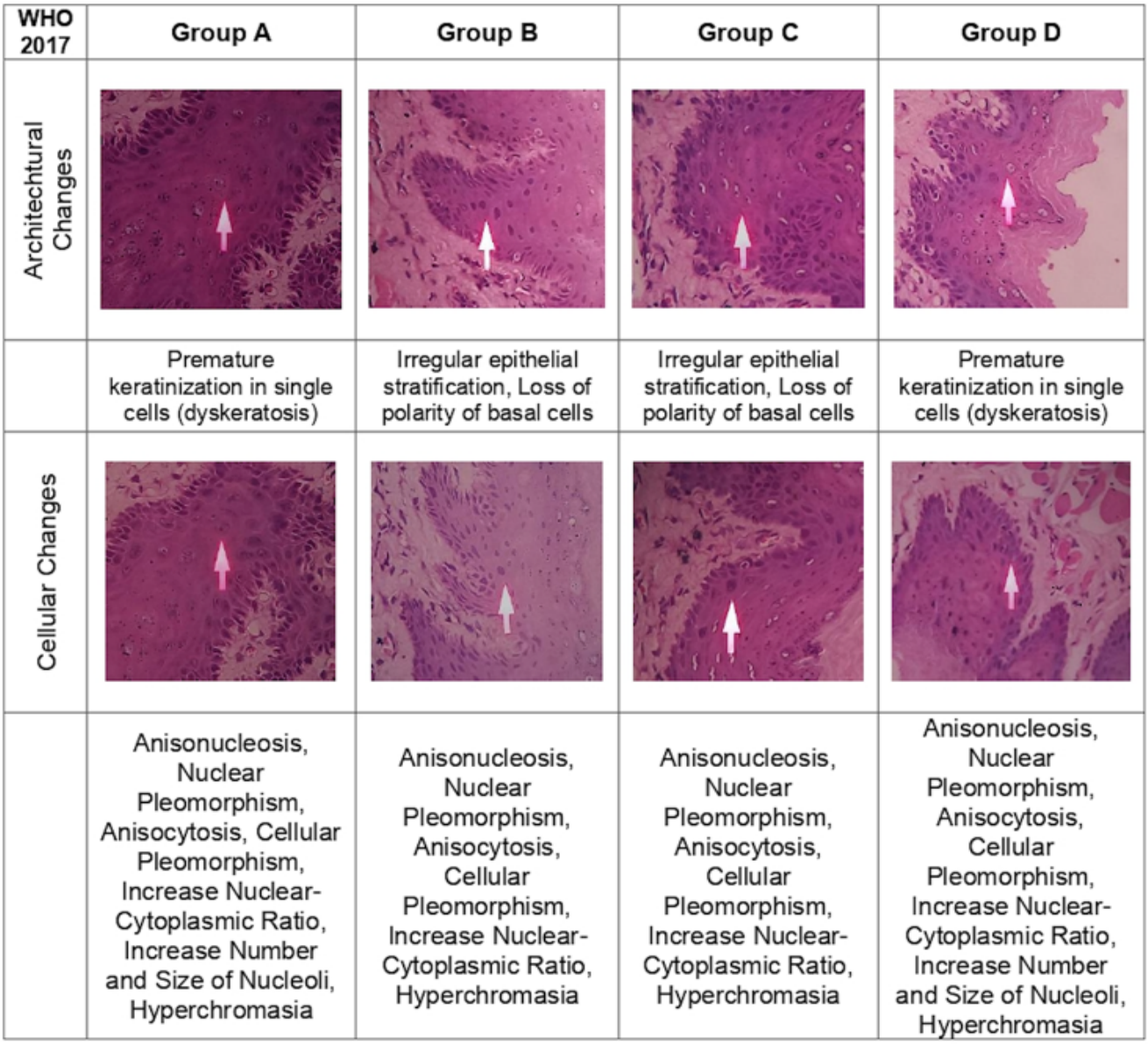

Figure 2. Images of histology examination results of rat buccal mucosa samples in terms of architectural and cellular changes.

References

1. Salian V, Dinakar C, Shetty P, Ajila V. Etiological Trends in Oral Squamous Cell Carcinoma: A Retrospective Institutional Study. Cancer Translational Medicine 2016; 2(2): 33-6.
2. Frohwitter G, Zimmermann OL, Kreutzer K, Doll C, Rendenbach CM, Dommisch H *et al*. Oxidative and nitrosative stress in oral squamous cell carcinoma. Cells Tissues Organs 2020; 209: 120-27.
3. Oliveira PA, Colaco A, Chaves R, Guedes-Pinto H, De-La-Cruz PLF, Lopez C. . Chemical carcinogenesis. Anais da Academia Brasileira de Ciencias 2007; 79(4): 593–916.
4. Katakwar P, Metgud R, Naik S, Mittal R. Oxidative stress marker in oral cancer: A review. J Cancer Res and Therapeutics 2016; 12(2): 438–446.
5. Chahar A, Chahar N, Kabirai A, Jagriti G. Chemical carcinogenesis: A brief review on mechanism & metabolism. J Or Med, Or Surgery, Or Patho and Or Radio 2020; 6(3):120–24.
6. Molecular Pathogenesis of Oral Squamous Cell Carcinoma. IntechOpen. 2020: 1-17. doi: 10.5772/intechopen.85650
7. Rastogi V, Puri N, Mishra S, Arora S, Kaur G, Yadav L. An insight to oral epithelial dysplasia. Int J Head and Neck Surg 2013; 4(2):74-82.
8. Tanaka T, Tanaka M, Tanaka T. Oral Carcinogenesis and Oral Cancer Chemoprevention: A Review. Pathology Research International 2011: 1–10.
9. Ashwini Baliga K, Kini R, Naik V, Kini S. Chemopreventive Agents; Its Role in Oral Cancer Prevention. Int J Adv Heal Sci 2015; 2(1):23-7.
10. Siddiqui IA, Sanna V, Ahmad N, Sechi M, Mukhtar H. Resveratrol nanoformulation for cancer prevention and therapy. Ann N Y Acad Sci 2015; 40(1): 1–12.
11. Sutardi. The content of the active ingredients of *Centella asiatica* and its properties to enhance the body's immune system. Jur Litbang Pertanian 2016; 35(3): 121-30.
12. James J, Dubery I. Identification and quantification of triterpenoid centelloids in *Centella asiatica* (L.) Urban by densitometric TLC. J Planar Chromatography 2011; 24(1): 82–87.

13. Deo PN, Deshmukh R. Pathophysiology of keratinization. J Oral Maxillo Pathol 2018; 22(1):86-91.
14. Ermawati D E, Sasmito E, Mufrod, Esha ND P, Ni Putu UA, Anggi KD *et al*. Optimum Dose and Formulation of *Centella asiatica* L. Urban Extract Against Igg of Wistar Strain Male Mices Which Induced By Bcg Vaccine. J Food and Pharm Sci 2016; 4(3):5–9.
15. Mustika A, Rahaju AS, Sudjarwo SA, Agil M, Mertaniasih NM. Mechanism of cencella asiatica extract in increasing alveolar macrophages apoptosis in rat tuberculosis models. J Int Dent and Med Res 2021; 14(2):812-9.
16. Delbo M, Calapai G. Assessment report on *Centella asiatica* (L.) Urban, herba. London: European Medicines Agency, 2012: 1–44.
17. Singh S, Gautam A, Sharma A, Batra A. *Centella asiatica* (L.): A plant with immense medicinal potential but threatened. Int Pharm Sci Rev Res 2010; 4(2): 9–17.
18. Widowati, W. *et al*. 'Screening of Superoxide Dismutase Activity in Various Plants', *Jkm*, 2005; 5(1): 33–48.
19. Panche, A. N., Diwan, A. D. and Chandra, S. R. 'Flavonoids: An overview', Journal of Nutritional Science, 2016; 5(e47): 1–15. doi: 10.1017/jns.2016.41.
20. Al-Khayri JM, Sahana GR, Nagella P, Joseph BV, Alessa FM, Al-Mssallem MQ. Flavonoids as Potential Anti-Inflammatory Molecules: A Review. *Molecules*. 2022; 27(9):2901. https://doi.org/10.3390/molecules27092901