

Original Research Article

EVALUATION OF ANTIOXIDANT AND XANTHINE OXIDASE INHIBITORY POTENTIAL OF METHANOLIC LEAF EXTRACT OF *STEVIA REBAUDIANA*

Running Title: Evaluation of Xanthine oxidase inhibitory potential of *Stevia rebaudiana*

ABSTRACT

Background: *Stevia rebaudiana* is a shrub-like plant that belongs to the sunflower family and is commonly referred as stevia. It is 1000 times sweeter than sugar even at a very low concentration. Xanthine oxidase is an enzyme that generates oxygen species and catalyzes the production of uric acid from purine metabolism. Overproduction of uric acid results in a clinical condition called gout. The aim of this study is to explore the phytochemicals, antioxidant and xanthine oxidase inhibitory potential of methanolic leaf extract of *stevia rebaudiana*.

Methods: Methanolic leaf extract of *Stevia rebaudiana* was prepared by the Hot Percolation method. Phytochemical screening was done to analyse the presence of various phytochemicals. The leaf extract was tested for its antioxidant and xanthine oxidase inhibitory potentials. The data were analyzed statistically by a one-way analysis of variance (ANOVA) followed by Duncan's multiple range test was used to see the statistical significance among the groups. The results with the $p < 0.05$ level were considered to be statistically significant.

Results: It was observed that the methanolic leaf extract of *Stevia rebaudiana* has significant antioxidant potential (IC_{50} of = 310 $\mu\text{g/ml}$) as well as xanthine oxidase inhibitory potentials (IC_{50} of = 270 $\mu\text{g/ml}$) and the activity increased in a dose dependent manner as compared to that of standard (Vitamin C and Allopurinol respectively).

Conclusion: The study proves the antioxidant and xanthine oxidase inhibitory efficacy of *Stevia rebaudiana* and throws light on the prospects of drug formation against oxidant activity and gout formation.

Keywords: *Stevia rebaudiana*, xanthine oxidase, allopurinol, antioxidant, Innovative technology, Novel method

INTRODUCTION

The WHO has estimated that 80% of the world's population meets their primary health care needs by the traditional medicines which came through their ancestors(1). Plant products are used as the predominant and cost effective source of medicine throughout the world for treating various human ailments(2).

Stevia rebaudiana is a perennial shrub belonging to the family-Asteraceae. This plant is native to Paraguay, Brazil and Argentina(3). The leaves of the plant have been used by indigenous people for centuries in medicines(4) and for formation of sweeter drinks such as green herbal tea(5). Japan was the first country to commercialize and use crude, unpurified extract. It is also a high demanding antidiabetic medicinal plant in Ayurvedic medicinal fields(6). *Stevia* and its extract have been studied widely from a sweetener point of view, however, a search through literature shows no information on the non-sweetening components, which make up about 80- 90% dry weight of this plant(7).

Leaves of *Stevia rebaudiana* exhibit effects on certain physiological systems such as the cardiovascular systems and influences in hypertension and hyperglycemia. Since, these activities can be correlated with the presence of antioxidant compounds in it, the leaf and callus extracts of *Stevia rebaudiana* were evaluated for their phenols, flavonoids' components and their total antioxidant activity.

Plants have the ability to execute important biological functions by producing naturally occurring chemical compounds for their normal metabolic activity commonly in them which are known as phytochemicals. Phytochemicals known as metabolites present in the plants which play a vital role in these processes.(8) The main sweet component present in the leaves of *Stevia rebaudiana* is Stevioside and other components are alkaloids, sterols, phenols, triterpenes, flavonoids, amino acids, β - carotenes, ascorbic acids and trace elements which introduces a definite physiological action on the human body by treating different diseases such as

hypertension, diabetes, cancer, neural disorders and cardiovascular disease. The fresh leaves of this plant have a nice liquorice taste.

Comment [g1]: Reference

Many antioxidant compounds are found in fruits and vegetables such as phenolics, carotenoids, anthocyanins and tocopherols. Free radicals have a significant role in processes of chemical materials degradation and also contribute to more human disorders. These also give protection to living organisms from damage caused by uncontrolled production of oxidative stress, concomitant lipid peroxidation and protein damage.(8),(10),(11)

Gout is a type of arthritis that causes painful inflammation in one or more joints, due to accumulation of uric acid. Antigout are used to treat gout and musculoskeletal disorder and form colchicine of anti inflammatory medication.(12),(13) Xanthine oxidase is a type of enzyme that generates free radical species and catalyse the oxidation of hypoxanthine to xanthine and oxidation of xanthine to uric acid (9)(14),(15)and it is generally treated with the standard drugs allopurinol. Allopurinol is a structural isomer of hypoxanthine and is an inhibitor of the enzyme xanthine oxidase.Our team has extensive knowledge and research experience that has translate into high quality publications (16),(17),(18),(19),(20),(21),(22),(23),(24), (25),(26) ,(27), (28), (29).The aim of the study is to evaluate the antioxidant and xanthine oxidase inhibitory potential of methanolic leaf extract of *Stevia rebaudiana*.

MATERIALS AND METHODS

1. Preparation of Methanolic leaf extract of *Stevia rebaudiana* :

Stevia rebaudiana was purchased from a nursery in Chennai. The leaves were collected , washed, crushed and made into powder. Methanol was added.80% of methanolic extract was obtained. The extract was prepared by a hot percolation method. Antioxidant and Anti gout potential of the extract was evaluated.

2. Phytochemical Screening test:

Test for phlobatannin:

1ml of the extract was treated with 1ml of 1% HCl and boiled for 10 mins. The formation of red color precipitate indicates the presence of phlobatannin.

Test for Carbohydrates:

Three to five drops of Molisch reagent was added with 1 mL of the extract and then 1 mL of concentrated sulphuric acid was added carefully through the side of the test tube. The mixture was then allowed to stand for two minutes and diluted with 5 mL of distilled water. The

development of a red or dull violet ring at the junction of the liquids showed the presence of carbohydrates.

Test for Flavonoids:

Few drops of 1% liquid ammonia were taken in a test tube and along with it 1ml of the extract was added resulting in the formation of yellow color thereby indicating the presence of flavonoids.

Test for Alkaloids:

2ml of sample was mixed with 2ml of HCl. Then 6 drops of HCN was added and further 2 drops of picric acid was added that resulted in a creamish pale yellow ppt indicating the presence of alkaloids.

Test for Terpenoids:

2 ml of sample along with 2ml of chloroform and 3ml of con. H₂SO₄ was added. Red color ppt obtained indicates the presence of terpenoids.

Test for proteins:

One milliliter of ninhydrin was dissolved in 1 mL of acetone and then a small amount of extract was added with ninhydrin. The formation of purple colour revealed the presence of protein.

Detection of saponins:

Foam test: A fraction of the extract was vigorously shaken with water and observed for persistent foam.

Test for steroids:

One ml of chloroform was mixed with 1 mL of extract and then ten drops of acetic anhydride and five drops of concentrated sulphuric acid were added and mixed. The formation of dark red colour or dark pink colour indicates the presence of steroids.

3. In vitro antioxidant activity (DPPH free radical scavenging activity)

Scavenging of 2, 2-Diphenyl-1-picrylhydrazyl (DPPH) radical was assessed by the method of Hatano et al, (1989). DPPH solution (1.0 ml) was added to 1.0 ml of extract at different concentrations (0.1 to 0.5mg/ml). The mixture was kept at room temperature for 50

minutes and the activity was measured at 517nm. Ascorbic acid at the same concentrations was used as standard. The capability to scavenge the DPPH radical was calculated and expressed in percentage (%) using following formula:

$$\text{DPPH radical scavenging (\%)} = \frac{\text{Control OD} - \text{Sample OD}}{\text{Control OD}} \times 100$$

4. In Vitro Xanthine Oxidase Inhibitory Activity of Methanolic leaf extract of *Stevia rebaudiana*:

In vitro Xanthine oxidase inhibitory of the extract was assessed as per the method of (Nguyen et al, 2004; Umamaheswari et al., 2007). Briefly, the assay mixture consisted of 1 ml of the fraction (0.1 to 0.5g/ml), 2.9 ml of phosphate buffer (pH 7.5) and 0.1 ml of xanthine oxidase enzyme solution (0.1 units/ml in phosphate buffer, pH 7.5), which was prepared immediately before use. After preincubation at 25°C for 15 min, the reaction was initiated by the addition of 2 ml of the substrate solution (150 M xanthine in the same buffer). The assay mixture was incubated at 25°C for 30 min. The reaction was then stopped by the addition of 1 ml of 1N hydrochloric acid and the absorbance was measured at 290 nm using a UV spectrophotometer. Allopurinol (0.1 to 0.5mg/ml), a known inhibitor of XO, was used as the positive control. One unit of XO is defined as the amount of enzyme required to produce 1 mmol of uric acid/min at 25°C. XO activity was expressed as the percentage inhibition of XO in the above assay system calculated as percentage of inhibition as follows.

Inhibitory activity (%) = (1 - As/Ac) x100 Where,

As – absorbance in presence of test substance, Ac – absorbance of control

5. Statistical analysis:

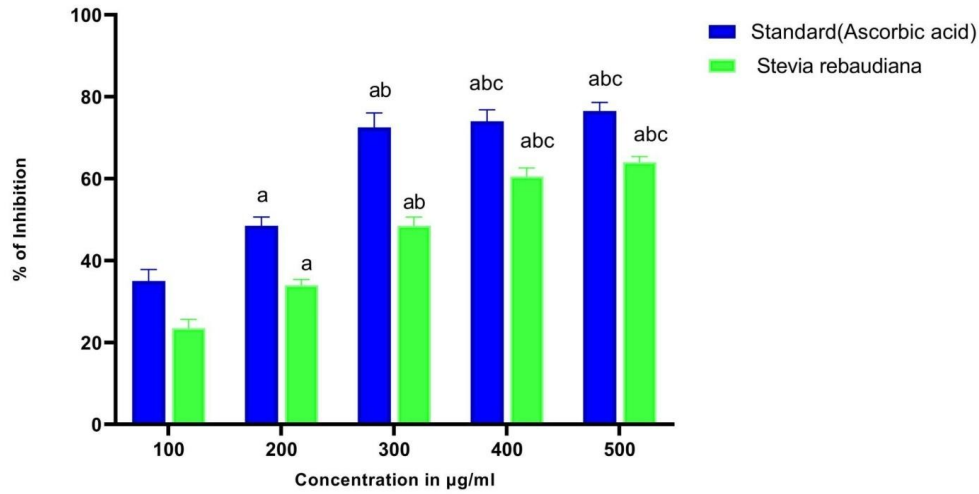
The data were subjected to statistical analysis using two-way analysis of variance (ANOVA) and Tukey's multiple range test to assess the significance of individual variations between the groups. In Tukey's test, significance was considered at the level of $p < 0.05$.

RESULTS

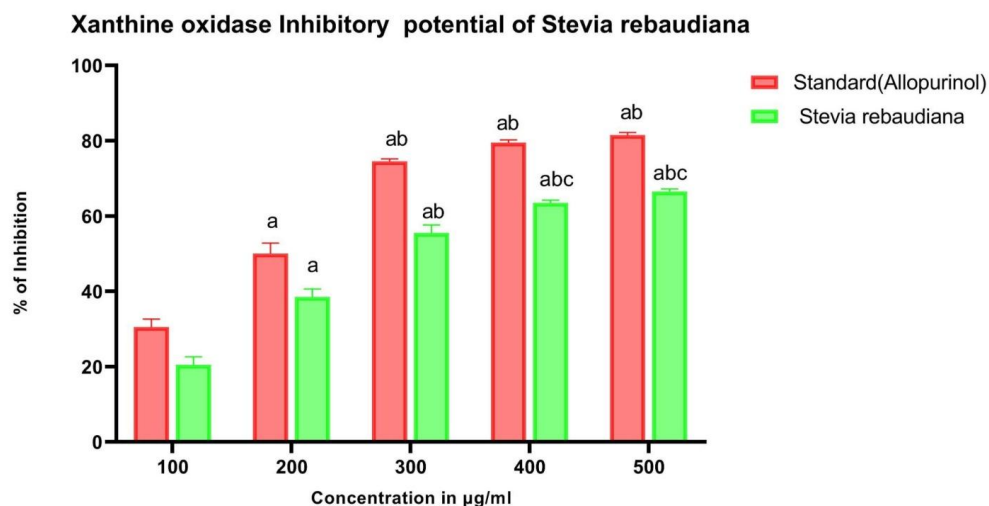
Table 1: Phytochemical Analysis of methanolic Leaf extract of *Stevia rebaudiana*

Phytochemicals	Methanolic Leaf extract of <i>Stevia rebaudiana</i>
Amino Acid	-
Flavonoids	+
Alkaloids	++
Terpenoids	+
Steroids	+
Carbohydrates	-

Antioxidant potential of Methanolic leaf extract of *Stevia rebaudiana*



Graph 1: The graph represents antioxidant potential of methanolic leaf extract of *Stevia rebaudiana* by DPPH Assay. X axis represents the concentration in µg/ml and Y axis represents the inhibitory potential of the extracts. Blue bar indicates standard- ascorbic acid, green represents methanolic leaf extract of *Stevia rebaudiana*. Each line represents Mean± SEM of 3 independent observations. Significance at $p < 0.05$ each.



Graph 2: The graph represents xanthine oxidase inhibitory potential of methanolic leaf extract of *Stevia rebaudiana*. X axis represents the concentration in µg/ml and Y axis represents the inhibitory potential of the extracts. Pink bar indicates standard- ascorbic acid, green represents methanolic leaf extract of *Stevia rebaudiana*. Each line represents Mean± SEM of 3 independent observations. Significance at $p < 0.05$ each.

DISCUSSION

The results revealed the strong presence of phytochemicals such as proteins, flavonoids, alkaloids, terpenoids, saponins and steroids (Table 1). Phytochemicals are secondary metabolites which are present only among plants (30). They possess various biologically active compounds that protect and help in the normal functioning of the human body. The Presence of phytochemicals like alkaloids, saponins, glycosides, proteins, and tannins indicates that the extract is potential for further in vitro analysis like antioxidant and anti gout activity. Bashyal et al., 2017 reported the relation between the strong antioxidant potential and the presence of

phytochemicals such as alkaloids, saponins, glycosides, proteins and tannins in their research study (31)

Antioxidant activity of aqueous leaf extract of *Stevia rebaudiana* was determined by performing DPPH free radical scavenging assay. Free radicals are molecules that possess an unpaired electron emerging in oxidative stress. Phenolic compounds have great importance in scavenging free radicals. The effect of antioxidant on DPPH free radical scavenging ability was measured as its antioxidant potential. The result obtained in this study shows that aqueous *Stevia rebaudiana* shows significant antioxidant ability as compared with vitamin c, with an Ic_{50} of 380 $\mu\text{g/ml}$. Further studies may be needed to ascertain the essential health benefits of the extract in prevention and accumulation of free radicals. Abbas et al., 2020 reported in their findings that methanol extract of arafaction with rich flavonoids can scavenge free radical significantly. (32).

A dose dependent xanthine oxidase inhibitory activity (anti gout) was observed for the extract in the current study ($Ic_{50} = 270\mu\text{g/ml}$) as compared to the standard drug-Allopurinol, The control exhibits greater percentage of inhibition of xanthine oxidase than the extract with the same concentration.

Studies of Bursac et al 2018 (33) has proved anti-gout potential in various herbal extracts which correlated with their rich phytochemical constituents. The results revealed that the standard drug Allopurinol is the most potential drug compared to the extract. In future, bioactive molecules from the extract can be purified to increase its anti-gout activity.

The current study has related the strong presence of aromatic phytochemicals to anti gout potential of the plant extract. Many research has been focussed to explore the anti gout potential of herbal extracts to replace synthetic anti gout drugs as it exhibits various side effects in the long run.

CONCLUSION

The study proved that *Stevia rebaudiana*, which has been used as a natural sweetener, is effective in inhibiting the activity of xanthine oxidase enzyme, and possesses in vitro antioxidant activity. Hence the study established the antigout activity of the extract, which can be used to formulate a therapeutic drug against gout if detailed investigations were conducted on this plant.

REFERENCES

1. Rajput SB, Tonge MB, Mohan Karuppaiyl S. An overview on traditional uses and pharmacological profile of *Acorus calamus* Linn. (Sweet flag) and other *Acorus* species [Internet]. Vol. 21, *Phytomedicine*. 2014. p. 268–76. Available from: <http://dx.doi.org/10.1016/j.phymed.2013.09.020>
2. Saxena M, Shakya AK, Sharma N, Shrivastava S, Shukla S. Therapeutic Efficacy of *Rosa damascena* Mill. on Acetaminophen-Induced Oxidative Stress in Albino Rats [Internet]. Vol. 31, *Journal of Environmental Pathology, Toxicology and Oncology*. 2012. p. 193–201. Available from: <http://dx.doi.org/10.1615/jenvironpatholtoxicoloncol.v31.i3.10>
3. Brandle JE, Starratt AN, Gijzen M. *Stevia rebaudiana*: Its agricultural, biological, and chemical properties [Internet]. Vol. 78, *Canadian Journal of Plant Science*. 1998. p. 527–36. Available from: <http://dx.doi.org/10.4141/p97-114>
4. Carakostas MC, Curry LL, Boileau AC, Brusick DJ. Overview: the history, technical function and safety of rebaudioside A, a naturally occurring steviol glycoside, for use in food and beverages. *Food Chem Toxicol*. 2008 Jul;46 Suppl 7:S1–10.
5. Mohan S, Hamuro M, Sorescu GP, Koyoma K, Sprague EA, Jo H, et al. I κ B α -dependent regulation of low-shear flow-induced NF- κ B activity: role of nitric oxide [Internet]. Vol. 284, *American Journal of Physiology-Cell Physiology*. 2003. p. C1039–47. Available from: <http://dx.doi.org/10.1152/ajpcell.00464.2001>
6. A KP, Viraj S. AMLAPITTA (HYPERACIDITY) - INTEGRATED OUTLINE AND AYURVEDIC MANAGEMENT [Internet]. Vol. 8, *Unique Journal of Ayurvedic and Herbal Medicines*. 2020. p. 4–7. Available from: <http://dx.doi.org/10.46791/ujahm.2020.080102>
7. Mahajan M, Thakur BK, Pal PK. Moisture stress and nitrogen availability modulate the secondary metabolite profiles, enzymatic activity, and physiological and agronomic traits of *Stevia rebaudiana*. *Plant Physiol Biochem*. 2021 Feb 20;162:56–68.

8. Muchtaromah B, Ahmad M, S EK, A YM, A VL. Phytochemicals, Antioxidant and Antifungal Properties of *Acorus calamus*, *Curcuma mangga*, and *Allium sativum* [Internet]. Vol. 3, KnE Life Sciences. 2017. p. 93. Available from: <http://dx.doi.org/10.18502/kls.v3i6.1119>
9. Chatsudthipong V, Muanprasat C. Stevioside and related compounds: therapeutic benefits beyond sweetness. *Pharmacol Ther*. 2009 Jan;121(1):41–54.
10. Wu F, Zhu J, Li G, Wang J, Veeraraghavan VP, Krishna Mohan S, et al. Biologically synthesized green gold nanoparticles from Siberian ginseng induce growth-inhibitory effect on melanoma cells (B16). *Artif Cells Nanomed Biotechnol*. 2019 Dec;47(1):3297–305.
11. Chen F, Tang Y, Sun Y, Veeraraghavan VP, Mohan SK, Cui C. 6-shogaol, a active constituents of ginger prevents UVB radiation mediated inflammation and oxidative stress through modulating Nrf2 signaling in human epidermal keratinocytes (HaCaT cells). *J Photochem Photobiol B*. 2019 Aug;197:111518.
12. Li Z, Veeraraghavan VP, Mohan SK, Bolla SR, Lakshmanan H, Kumaran S, et al. Apoptotic induction and anti-metastatic activity of eugenol encapsulated chitosan nanopolymer on rat glioma C6 cells via alleviating the MMP signaling pathway [Internet]. Vol. 203, *Journal of Photochemistry and Photobiology B: Biology*. 2020. p. 111773. Available from: <http://dx.doi.org/10.1016/j.jphotobiol.2019.111773>
13. Babu S, Jayaraman S. An update on β -sitosterol: A potential herbal nutraceutical for diabetic management. *Biomed Pharmacother*. 2020 Nov;131:110702.
14. Malaikolundhan H, Mookkan G, Krishnamoorthi G, Matheswaran N, Alsawalha M, Veeraraghavan VP, et al. Anticarcinogenic effect of gold nanoparticles synthesized from *Albizia lebeck* on HCT-116 colon cancer cell lines. *Artif Cells Nanomed Biotechnol*. 2020 Dec;48(1):1206–13.
15. Han X, Jiang X, Guo L, Wang Y, Veeraraghavan VP, Krishna Mohan S, et al. Anticarcinogenic potential of gold nanoparticles synthesized from *Trichosanthes kirilowii* in colon cancer cells through the induction of apoptotic pathway. *Artif Cells Nanomed*

Biotechnol. 2019 Dec;47(1):3577–84.

16. Gothai S, Muniandy K, Gnanaraj C, Ibrahim IAA, Shahzad N, Al-Ghamdi SS, et al. Pharmacological insights into antioxidants against colorectal cancer: A detailed review of the possible mechanisms. *Biomed Pharmacother.* 2018 Nov;107:1514–22.
17. Veeraraghavan VP, Hussain S, Balakrishna JP, Dhawale L, Kullappan M, Ambrose JM, et al. A Comprehensive and Critical Review on Ethnopharmacological Importance of Desert Truffles: *Terfezia claveryi*, *Terfezia boudieri*, and *Tirmania nivea* [Internet]. *Food Reviews International.* 2021. p. 1–20. Available from: <http://dx.doi.org/10.1080/87559129.2021.1889581>
18. Sathya S, Ragul V, Veeraraghavan VP, Singh L, Niyas Ahamed MI. An in vitro study on hexavalent chromium [Cr(VI)] remediation using iron oxide nanoparticles based beads. *Environmental Nanotechnology, Monitoring & Management.* 2020 Dec 1;14:100333.
19. Yang Z, Pu M, Dong X, Ji F, Priya Veeraraghavan V, Yang H. Piperine loaded zinc oxide nanocomposite inhibits the PI3K/AKT/mTOR signaling pathway via attenuating the development of gastric carcinoma: In vitro and in vivo studies. *Arabian Journal of Chemistry.* 2020 May 1;13(5):5501–16.
20. Rajendran P, Alzahrani AM, Rengarajan T, Veeraraghavan VP, Krishna Mohan S. Consumption of reused vegetable oil intensifies BRCA1 mutations. *Crit Rev Food Sci Nutr.* 2020 Oct 27;1–8.
21. Barma MD, Muthupandiyani I, Samuel SR, Amaechi BT. Inhibition of *Streptococcus mutans*, antioxidant property and cytotoxicity of novel nano-zinc oxide varnish. *Arch Oral Biol.* 2021 Jun;126:105132.
22. Samuel SR. Can 5-year-olds sensibly self-report the impact of developmental enamel defects on their quality of life? *Int J Paediatr Dent.* 2021 Mar;31(2):285–6.
23. Samuel SR, Kuduruthullah S, Khair AMB, Shayeb MA, Elkaseh A, Varma SR. Dental pain, parental SARS-CoV-2 fear and distress on quality of life of 2 to 6 year-old children during COVID-19. *Int J Paediatr Dent.* 2021 May;31(3):436–41.

24. Tang Y, Rajendran P, Veeraraghavan VP, Hussain S, Balakrishna JP, Chinnathambi A, et al. Osteogenic differentiation and mineralization potential of zinc oxide nanoparticles from *Scutellaria baicalensis* on human osteoblast-like MG-63 cells [Internet]. Vol. 119, Materials Science and Engineering: C. 2021. p. 111656. Available from: <http://dx.doi.org/10.1016/j.msec.2020.111656>
25. Yin Z, Yang Y, Guo T, Veeraraghavan VP, Wang X. Potential chemotherapeutic effect of betalain against human non-small cell lung cancer through PI3K/Akt/mTOR signaling pathway. *Environ Toxicol*. 2021 Jun;36(6):1011–20.
26. Veeraraghavan VP, Periadurai ND, Karunakaran T, Hussain S, Surapaneni KM, Jiao X. Green synthesis of silver nanoparticles from aqueous extract of *Scutellaria barbata* and coating on the cotton fabric for antimicrobial applications and wound healing activity in fibroblast cells (L929). *Saudi J Biol Sci*. 2021 Jul;28(7):3633–40.
27. Mickymaray S, Alfaiz FA, Paramasivam A, Veeraraghavan VP, Periadurai ND, Surapaneni KM, et al. Rhaponticin suppresses osteosarcoma through the inhibition of PI3K-Akt-mTOR pathway. *Saudi J Biol Sci*. 2021 Jul;28(7):3641–9.
28. Teja KV, Ramesh S. Is a filled lateral canal – A sign of superiority? [Internet]. Vol. 15, Journal of Dental Sciences. 2020. p. 562–3. Available from: <http://dx.doi.org/10.1016/j.jds.2020.02.009>
29. Kadanakuppe S, Hiremath S. Social and Behavioural Factors Associated with Dental Caries Experience among Adolescent School Children in Bengaluru City, India [Internet]. Vol. 14, British Journal of Medicine and Medical Research. 2016. p. 1–10. Available from: <http://dx.doi.org/10.9734/bjmmr/2016/24021>
30. ul-Ferdous MR-. POTENTIAL IN-VITRO CARDIOPROTECTIVE ASSAY AND PHYTOCHEMICALS SCREENING OF EUPHORBIA HIRTA (LNN) [Internet]. World Journal of Pharmaceutical Research. 2017. p. 92–9. Available from: <http://dx.doi.org/10.20959/wjpr20174-8105>
31. Bashyal S, Rai S, Osama Abdul Manan | Faiz Hashmi | Avijit Guha, IILM-College of

Engineering and Technology. Preliminary Phytochemical Testing and Antimicrobial activity of *Calotropis procera* leaves [Internet]. Vol. -2, International Journal of Trend in Scientific Research and Development. 2017. p. 926–30. Available from: <http://dx.doi.org/10.31142/ijtsrd7104>

32. Abbas A, Hassan SS-U, Sharif A, Ahmed S. Evaluation of the antioxidant and anti-inflammatory activities of solvent extracts of (*Boiss*) Rech. f. whole plant. *Nat Prod Res.* 2020 Feb;34(4):575–9.
33. Bursać Kovačević D, Maras M, Barba FJ, Granato D, Roohinejad S, Mallikarjunan K, et al. Innovative technologies for the recovery of phytochemicals from *Stevia rebaudiana* Bertoni leaves: A review. *Food Chem.* 2018 Dec 1;268:513–21.

UNDER PEER REVIEW