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Lehninger, A.L., Nelson, D.L. and Cox, M.M. (2004). Lehninger Principles of Biochemistry, (4th edition), W.H. Freeman & Co., New York, USA, pp. 73-111.

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Morphological and Physico-biochemical Characterization of Tomato Plant using Different Waste Vermicompost by Earthworm *Eudrilus eugeniae*

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Abstract

Vermicomposting is an environmentally friendly and cost-effective process for decomposing organic waste. Paper mill sludge waste (PW) and sugar mill press mud waste (SPW) are often indiscriminately discarded into the environment, causing health hazards and environmental pollution. Due to its high nitrogen content, this waste can be turned into organic fertilizer to reduce pollution and provide an inexpensive raw material for fertilizer production. Four compost mixtures (E1, E2, E3 and E4) were prepared in different proportions. PW and SPW with CD + PW + SPW cow manure control mix. After 90 days of composting, the nutritional composition of the compost was analyzed and the vermicompost obtained was applied to *Solanum lycopersicum*. The pH, N, P, K, C: N, and Total Organic Carbon (TOC) of the vermicompost were chemically analyzed. Randomly selected seedlings from each treatment were transplanted into pots, and various parameters such as Plant growth, leaf chlorophyll content, mineral concentrations, fruit characteristics, yield and fruit quality (including color, pH, ascorbic acid, titratable acidity and total solids) soluble) **is** evaluated. Most of the growth, yield**,** and quality indicators increased quite well compared to the control, but the difference between the treatments was not significant. In conclusion, vermicomposting of PW and SPW resulted in the production of high-quality organic fertilizer that can improve plant growth, yield, and quality. This can help reduce pollution and minimize the need for additional nitrogen sources, thereby reducing costs. The findings of this study suggest that vermicomposting can be a useful tool for sustainable waste management and agricultural practices.

Key words: Paper mill waste, β-Carotene, Lycopene, Total chlorophyll, Total yield

Introduction

Paper mill industries play an important role in the global economic environment and people's ability to make a living. With the passage of time, it was discovered that there had been a significant increase in global paper production. For the time being, the United States is the world's biggest paper producer. However, the Confederation of the European Paper Industry predicts that global paper demand will exceed 500 million tons by 2025 (1). India produces 2.6% of the world's paper, contributing to the country's economic development (2). Due to the high organic matter content, developing countries like India face major barriers in the area of proper waste management (3,4,5). Traditional sugar sludge management methods, including

composting, land filling, incineration, pollute the soil and impact global warming by generating harmful gases. Water pollution due to improper disposal of domestic and industrial waste is also a serious problem in this country (6).

Large volumes of sludge from paper mills and sugar mills cause environmental problems, polluting air, water and soil ecosystems. One of the essential techniques for valorization is vermi-technology, a biotechnological invention that holds tremendous promise for the sustainable management of wastes by bio-converting solid organic wastes into a valuable molecule under favorable ecological conditions (7,8). Most reassuringly, it maintains the region's economy and promotes livelihood in rural areas. It outlines a good biodegradation technique in which earthworms and microorganisms work together to cause severe deformation in organic solid wastes. (9,10). In contrast, soil bacteria ferment them into organic nitrogen that can be used for agricultural purposes. Population growth coupled with inadequate food supplies, declining quality of health care, high unemployment and increasing environmental degradation are some of the key underlying issues affecting global prosperity. Around the world. the future of "humanity". These problems are expected to increase in severity as the world population continues to farm (11).

Tomato (*Solanum lycoperscium*) is a major vegetable crop grown around the world. Lycopene, the main pigment responsible for the red color of tomatoes, is one of the most abundant natural carotenoids found in tomatoes (90%) (12). It is a commercial vegetable grown all over India due to its high nutritional value and reliable price. India ranks first in area (5.1 million hectares) and production (880,000 tons). It has ayurvedic medicinal properties and is known to benefit diabetics (13). Carotenoids such as carotene and lycopene play important roles in antioxidant protection against lipid peroxidation in living cells (14). The unsaturated open-chain carotenoid of lycopene has 11 covalent double bonds and is an effective free radical scavenger (15) also they are important dietary sources of vitamin A. After bioconversion of retinol-carotene to provitamin A (16). Therefore, the introduction of these biodegradable materials into the soil not only increases the organic matter content and soil fertility, but also increases the microbial activity (17,18,19,20). Improvements in soil fertility and microbial activity due to the return of crop wastes such as compost improve farmland health and increase root strength and biological properties. plant physiology such as photosynthesis rate, chlorophyll and carbohydrate content. (21,22). However, it was discovered that using organic fertilizer along with fertilizers high in nitrogen, phosphate, and potassium was more beneficial for increasing production and supplying macronutrients to tomato plants.

The goal of the current research was to handle waste from sugar and paper mills using a combination of innovative vermicompost method techniques. Evaluation of the effect of treated waste on the content of β-carotene, lycopene and chlorophyll in tomato fruit and growth of the plant.

Materials and Methods

Raw material collection

Paper mill sludge waste is collected from TNPL in Pugalur, Karur district, Tamil Nadu. Sugar mill press mud waste is collected from Mohanur sugar mill industry. Cow dung were also collected at the same location. The collected waste was washed several times with tap water, dried in the sun and used in composting experiments.

Experimental setup

Raw materials (CD:SW:PW) are put into 5 round barrels (25cm diameter, 40cm high) and mixed in different ratios C (4:0:0), E1 (4:2:2) and E2 . (4:3:1), E3 (4:1:3) and E4 (4:4:4). Compost moisture was maintained at 40-60% throughout the experimental period (90 days). Five types

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of compost were introduced into the soil 3 days before sowing at the rate of 30 kg per field (25 sowing). General stages such as irrigation, weeding, pest control are carried out according to the process. This study was conducted on tomato plants (*Solanum lycopersicum*) grown on sandy soil combined with drip irrigation system. Tomato seeds were sown in seedling trays in June 2021 and placed in a greenhouse. Healthy seedlings of 30 days old were planted in plots of 15 m^2. Seedlings were planted in vertical rows (25 plants per row) with 1.0 m row spacing and 0.5 m row spacing, experimental setup consisting of completely randomized plots and 5 composting treatments. . Each formula was repeated 5 times. Each compost treatment was separated from each block by 1 m grooves. Each plot is 10 m long, including 25 trees, of which 10 central trees are selected to determine growth characteristics, yield, and fruit quality.

Physico-chemical analysis

Use DH2O dual organic fertilizer solution at the ratio of 1:10 (w/v) and analyze the results with a digital pH meter (23), The pH of compost sample was determined. Total organic carbon (TOC) was calculated using (24).The Micro Kjeldahl technique was used to determine Total Nitrogen (TN) (25, 26). A colorimetric method was used to determine total phosphorus (TP) (27). After digesting the sample in an acidic mixture (HNO3 concentrate; concentrated HClO4, 4:1, v/v), total potassium (TK) was measured using a flame photometer (28).

Morphological parameters

The following morphological data were recorded: plant height (cm), fruit diameter (cm), fruit weight (g) and total yield (g/plant) (29).

Biochemical analysis of fruit pigments

10-20 ml of acetone-hexane (4:6) solvent was used to homogenize 1 g of tomato fruit. After homogenization, the supernatant was used for biochemical analysis (30).

Amount of lycopene, β-carotene and chlorophyll

The amounts of lycopene, beta-carotene and chlorophyll were determined using the analytical method and technique described by the technique of Mackinney and Kimura. For lycopene, β-carotene and other nutrients, chlorophyll a, b and total are expressed in mg/100 ml.

Fruit quality characteristics

10 fruit samples were previously used to evaluate fruit quality characteristics. Fruit was cut into small pieces and tomato juice from each of the 10 fruit samples was extracted by measured volume (ml) using a juicer. Pure juice is used for quality control. After removing the seeds, skin and pulp, the volume of juice was measured in a graduated cylinder (31).

pH

In 50 ml of filtrate containing 10 g of pulp mixed in 100 ml of distilled water, determine the pH of tomato juice (32).

Titrated acidity (TA)

Titration of 10 g of homogenized tomato juice sample after dilution with 50 ml of distilled water, 0.1% NaOH solution **at** pH 8.17 was used to determine the acidity (33) and the results are reported in g/L.

Ascorbic acid (AA)

The concentration of ascorbic acid in selected tomatoes was determined by the method of (34). The procedure consisted of homogenizing a mixture of fruit pulp (5 g) and 5 ml of 0.1% HCl (w/v), then centrifuging the mixture at 10,000 rpm for 10 min and accumulating supernatant into the condenser. The absorbance of the supernatant at 243 nm was then evaluated using a spectrophotometer.

Statistical analysis

Statistical analyzes were performed using one-way ANOVA and Duncan's multiple test

(DMRT) in SPSS (version 21) and the data were compared.

Results and Discussion

90 days after transplanting, tomato plant height responded significantly to 5 different treatments. The results of physical and chemical testing of the final product are shown in (Table 1). The pH level is one of the most important aspects of the composting process. The pH level is reduced as much as possible during the E2 treatment. The maximum available nitrogen content of E2 was 7.33%, which is consistent with the conclusion (35). Nitrogen is an essential component of amino acids, the basic structural unit of proteins. At the same time, due to the ammonification process, the nitrogen content in the compost is significantly reduced, leading to the conversion of a part of organic NH3 into NH4+ ions (36). Phosphorus provides energy for plant growth and maintains plant balance (37). The waste compost in our present study contained less phosphorus than the control (0.26%). Phosphorus increases the amount of chlorophyll in plants. E2 has a gradually increasing potassium (K) content (4.40%). Wool and feather waste can provide nutrients (N, P, K) to plants and improve soil biology and chemistry (38). A significant increase in plant height was observed with an organic fertilizer rich in E3 (67.64 cm) and then treated with E2 (66.25 cm). However, the shortest plants were found in the control variant (58.38 cm) (Table 2). These results may be related to the physico-chemical state of the palm waste modified by the addition of water and fertilizer for plant growth (39). All tomatoes showed a significant difference. Plant height is a significant component, according to (40), because it has the most beneficial effect on fruit yield. Fruit weight, volume, and therefore total fruit production were highest in vermicompost enhanced with a greater rate of E2, while control yield was lowest. (E4 and C) gave the lowest values of total yield and its components. In terms of average fruit weight and diameter, E2 produced the highest values (2.197 kg) and (1.283 kg), respectively, when compared to C. In general, all compost treatments with differing waste ratios produced significantly higher yields and components than control soil. These findings support the findings of (41) who found that adding cow dung vermicompost to tomato plants increased plant growth and yield while decreasing element uptake, primarily N, P, and K. In addition, compost enriched with different vermicompost ratios (E1 to E4) significantly increased fruit length and diameter compared with enriched control compost. The high yield and nutrient concentration of tomato plants fertilized with organic fertilizers added to cow manure may be due to the fact that these substances not only contain sufficient nutrients, but also because nutrients are slowly released into the soil. This minimizes nutrient loss and leaching while increasing nutrient utilization (42). Furthermore, compared with balanced chemical fertilizers, the use of organic fertilizers increases the organic carbon content and fertility of the soil, leading to a tendency for higher yields (43). The weight and number of fruits determine the yield of the plant. As a result, fruit weight is directly proportional to plant yield (44). The pigment composition of tomato fruit was determined and the results published (Table 3). The results showed that increasing the sulfur content resulted in a 44.5% higher lycopene content in the fruit for a tomato variety (45), with the highest lycopene content recorded being E2 (0.571 mg/100 ml).). , E1 (0.563 mg/100 ml), E4 (0.538 mg/100 ml), E3 (0.521 mg/100 ml) and the lowest C (0.472 mg/100 ml). Plants treated with E2 had higher β-carotene content than control plants (E2 (0.271 mg/100 ml), E1 (0.249 mg/100 ml), E4 (0.205 mg/100 ml), E3 (0.173 mg/100 ml). ml). ml), E4 (0.205 mg/100 ml), E3 (0.173 mg/100 ml) and control (0.147 mg/100 ml) (46). Tomatoes from control plants had 0.183 mg/100 ml of chlorophyll (a) and 0.338 mg/100 ml of total chlorophyll, while

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Table 1 Physicochemical parameter of feather compost samples

Treatments	pH	OC (%)	N (%)	P (%)	K (%)
C	8.5 ±0.52	0.85 ±0.02	1.02 ±0.14	0.20 ±0.02	1.95 ±0.53
E1	8.32 ±0.42	1.08 ±0.14	1.54 ±0.05	0.51 ±0.05	2.05 ±0.27
E2	8.09 ±0.27	1.41 ± 0.05	3.02 ±0.21	0.96 ±0.12	3.15 ±0.15
E3	8.15 ±0.36	1.30 ±0.21	2.68 ± 0.09	0.72 ± 0.15	2.74 ±0.36
E4	8.43 ±0.41	1.01 ±0.13	1.27 ±0.15	0.35 ±0.08	2.01 ±0.18

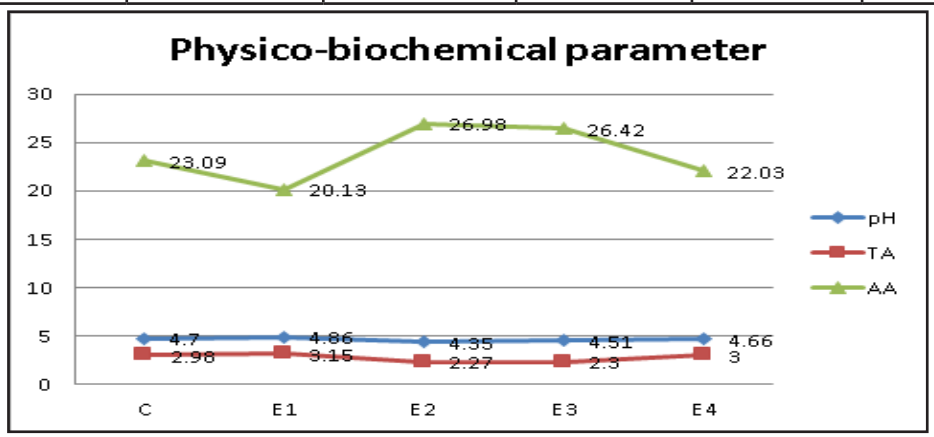
Table 2: Total yield parameters of tomato plants

E2 had the lowest chlorophyll (b) content of 0.227 mg/100 ml.

Treatment	Plant height at 90 days (cm)	Fruit diameter (cm)	Fruit weight (g)	No. of fruits/ plant	Total yield (Kg/ plant)
C	58.38 ± 1.26	2.61 ± 0.18	10.33 ± 0.85	45.62 ± 3.86	1.283±1.52
E1	61.71 ± 0.98	2.82 ± 0.27	11.33 ± 1.15	60.93 ± 3.55	1.483±0.96
E2	66.25 ± 1.52	3.79 ± 0.59	11.87 ± 0.92	71.13 ± 4.04	2.197 ± 1.36
E3	67.64 ± 1.81	3.88 ± 0.36	13.33 ± 1.23	62.15 ± 4.16	2.015 ± 2.04
E4	68.45 ± 1.20	3.74 ± 0.27	12.57 ± 1.28	59.17 ± 3.75	1.305 ± 2.18

Table 3: Analysis of plant pigments chlorophyll, lycopene and β-carotene

Experimental treatment	Chlorophyll a (mg/100 ml)	Chlorophyll b (mg/100 ml)	Total Chlorophyll (mg/100 ml)	Lycopene (mg/100 ml)	β-Carotene (mg/100 ml)
C	0.176	0.092	0.269	0.472	0.147
E1	0.150	0.143	0.305	0.563	0.249
E2	0.183	0.227	0.338	0.571	0.271
E3	0.182	0.105	0.287	0.521	0.173



E4	0.133	0.153	0.264	0.538	0.205

Figure 1: Physico-biochemical parameter of tomato fruit

Fruit quality characteristics

The results in Table 5 show that different compost combinations influenced the AA, TA, pH, and fruit juice (%) of tomatoes. Ascorbic acid is vital to one's diet because it will be cure chronic disease, scurvy, and stress. According to data reported in, the E2 had the greatest AA concentration (26.98 0.51 mg/100 g), followed by the E3 and the shortest control (23.09 0.15 mg/100 g) (Table 4). Light rates in tomatoes at the end of harvest, temperature conditions during pre-harvest, at harvest and after harvest, and changes in AA content for the same variety are reasonable explanation. (47). At maturity, the level of AA was higher, but it later decreased (48). In this investigation, the TA values ranged from 0.28 to 0.49%. The E1 compost had the highest TA value (0.45-0.56%), while E2 organic fertilizer has the lowest TA value (0.30-0.32%). These results contradict the conclusion of (49) who found that TA values were higher in plants treated with organic compounds compared with plants treated with fertilizers or controls. . The pH of the fruit is an important factor in the consumption of fresh tomatoes; The low pH improves the flavor of the fruit (50). All organic fertilizers have pH values that range from 4.35 to 4.86. These values are relatively similar to those reported by different researchers in previous studies (51, 52). They found pH values between 4.19 and 4.45 in many varieties of tomatoes grown on soils that had been improved with multiple applications of organic and mineral fertilizers.

Conclusion

This study found that using vermicompost made from paper and sugar mill waste as a fertilizer for tomato plants grown in the field can enhance plant growth and fruit quality by increasing the levels of photosynthetic pigments, vitamin C, lycopene, carotene, and other nutrients. The study evaluated different waste-to-waste ratios of vermicompost and found that tomato plants responded differently to each type of vermicompost. However, when vermicompost material was modified with high concentrations of paper mill waste and sugar press sludge (E2), significant differences in tomato fruit growth, yield and quality were observed compared to those amended with NPK chemical fertilizer and soil.The study concluded that no single nutrient source, such as chemical fertilizer, organic manure, or biofertilizer, can fully meet all nutrient requirements. However, vermicompost can increase the quantity and quality of nutrients, leading to quicker nutrient absorption and improving growth and yield parameters in crop plants.

References

1. Ganguly, R.K., Chakraborty, S.K., (2020a). Paper pulp mill wastes: A curse or boon – Modern approach of recycling. In: C., Hussain (Ed.), Handbook of Environmental Materials Management. Springer, Cham, http://dx.doi.org/10.1007/978-3-319-58538-3_216-1.

2. Ganguly, R.K., Chakraborty, S.K., (2020b). Eco-management of industrial organic wastes through the modified innovative vermicomposting process: A sustainable approach in tropical countries. In: Bhat, S., Vig, A., Li, F., Ravindran, B. (Eds.), Earthworm Assisted Remediation of Effluents and Wastes. Springer, Singapore, http://dx-.doi.org/10.1007/978-981-15-4522-1_10.

3. Elvira, C., Sampedro, L., Benitez, E., Nogales, R., (1998). Vermicomposting of sludges from paper mill and dairy industries with *Eisenia andrei*: a pilot scale study. Bioresour. Technol. 63, 205–211.

4. Ganguly, R.K., Chakraborty, S.K., (2018). Assessment of microbial roles in the bioconversion of paper mill sludge through vermicomposting. J. Environ. Health Sci.

Eng 6, 205–212.

5. Gupta, R., Garg, V.K., (2009). Vermiremediation and nutrient recovery of non-recyclable paper waste employing *Eisenia fetida*. J. Hazard. Mater. 162, 430–439.

6. Priyanka Kakkar, Neeraj Wadhwa., (2021). Utilization of Cellulase from Colocasia esculenta in Treatment of Cotton Fabric. Curr. Trends Biotechnol. Pharm. 6 (3) 407 – 416.

7. Suthar, S., (2008). Bioconversion of post harvest crop residues and cattle shed manure into value-added products using earthworm Eudriluseugeniae Kinberg. Ecol. Eng. 32, 206–214.

8. Hait, S., Tare, V., (2012). Transformation and availability of nutrients and heavy metals during integrated composting–vermicomposting of sewage sludges. Ecotoxicol. Env. Saf. 79, 214–224.

9. Sinha, R.K., Agarwal, S., Chauhan, K., Dalsukh, V., (2010). The wonders of earthworms & its vermicompost in farm production: Charles darwin's 'friends of farmers', with potential to replace destructive chemical fertilizers from agriculture. Agric. Sci. 1, 76–94.

10. Bhat, S.A., Singh, S., Singh, J., Kumar, S., Vig, A.P., (2018a). Bioremediation and detoxification of industrial wastes by earthworms: vermicompost as powerful crop nutrient in sustainable agriculture. Bioresour. Technol. 252, 172–179.

11. Sangeetha Menon1, Syeda Ayesha , Pavithra K, Malsawmtluangi., (2021). Growth and Nutritional Indices of Oyster Mushroom (Pleurotusostreatus) on Different Substrates. Current Trends in Biotechnology and PharmacyVol. 15 (5) 365-372.

12. Shi J, Le Maguer M, Kakuda Y, Liptay A, Niekamp F (1999) Lycopene degradation and isomerization in tomato dehydration. Food Res Int 32:15–21. https ://doi. org/10.1016/S0963 -9969(99)00059 -9.

13. Agarwal A, Prahakaran SA, Said TM (2005) Prevention of oxidative stress injury to sperm. J. Androl 26:653–660. https :// doi. org/10.2164/jandr ol.05016.

14. Shi J, Maguer M (2000) Lycopene in tomatoes: chemical and physical properties affected by food processing. Crit Rev Biotechnol 20:293–334. https ://doi. org/10.1080/07388 55009 11442 12.

15. Sivagamasundari1 U, Gandhi A., (2021). Effect of bacterial endophytes - Azospirillumbrasilense and Pseudomonas fluorescens on growth and yield of Brinjal var. Annamalai in field trial.Current Trends in Biotechnology and Pharmacy Vol. 15 (5) 489-495.

16. Tang G (2010) Bioconversion of dietary provitamin A carotenoids to vitamin A in human. Am J Clin Nutr 91:148S–1473S. https ://doi. org/10.3945/ajcn.2010.28674.

17. Zayed G, Abdel-Motaal H (2005) Bio-active composts from rice straw enriched with rock phosphate and their effect on the phosphorous nutrition and microbial community in rhizosphere of cowpea. Bioresource Technology 96: 929-935.

18. Benito M, Masaguer A, Moliner A, Antonio RD (2006) Chemical and physical properties of pruning waste compost and their seasonal variability. Bioresource Technology 97: 2071-2076.

19. Khan, AA, Bibi H, Ali Z, Sharif M, Shah SA, Ibadullah H, Khan K, Azeem I, Ali S (2017) Effect of compost and inorganic fertilizers on yield and quality of tomato. Academia Journal of Agricultural Research 5: 287-293.

20. Liu CH, Liu Y, Fan C, Kuang SZ (2013) The effects of composted pineapple residue return on soil properties and the growth and yield of pineapple. Journal of Soil Science and Plant Nutrition 13: 433-444.

21. Joshi D, Hooda KS, Bhatt JC, Mina BL, Gupta HS (2009) Suppressive effects of composts on soil-borne and foliar diseases of French bean in the field in the western Indian Himalayas. Crop Protection 28: 608-615.

22. Yogev A, Raviv M, Kritzman G, Hadar Y, Cohen R, Kirshner B, Katan J (2009) Suppression of bacterial canker of tomato by composts. Crop Protection 28: 97-103.

23. Vasanthi K, Chairman K, Ranjit Singh AJA (2014) Sugar factory waste vermicomposting with an epigeic earthworm, *Eudriluseugeniae*(Kinberg). Am J Drug Discov Dev 4:22–31. https ://doi. org/10.3923/ ajdd.2014.22.31.

24. Abdullah N, Chin NL (2010) Simplex-centroid mixture formulation for optimized composting of kitchen waste. Bioresour Technol. 101(21):8205–8210. https ://doi. org/10.1016/j.biort ech.2010.05.068.

25. Mohee R, Driver MFB, Sobratee N (2008) Transformation of spent broiler litter from exogenous matter to compost in a sub-tropical context. Bioresour Technol. 99(1):128–136. https ://doi. org/10.1016/j. biort ech.2006.11.040.

26. Unmar G, Mohee R (2008) Assessing the effect of biodegradable and degradable plastics on the composting of green wastes and compost quality. Bioresour Technol 99(1):6738–6744. https ://doi. org/10.1016/j.biort ech.2008.01.016.

27. John MK (1970) Colorimetric determination of phosphorus in soil and plant material with ascorbic acid. Soil Sci 109:214–220. https :// doi.org/10.1097/00010 694-19700 4000-00002.

28. Bansal S, Kapoor KK (2000) Vermicomposting of crop residues and cattle dung with *Eisenia foetida*. Bioresour Technol. 73:95–98. https ://doi.org/10.1016/S0960 -8524(99)00173 –X.

29. Shah, A.H., Munir, S.U., Noor-Ul-Amin, Shah, S.H., (2011). Evaluation of two nutrient solutions for growing tomatoes in a non-circulating hydroponics system. Sarhad J. Agric. 27, 557–567.

30. Nagata M, Yamashita I (1992) A simple method for simultaneous determination of chlorophyll and carotenoids in tomato fruit. J Jpn Soc Food Sci Technol 39(10):925–928. https ://doi.org/10.3136/ 1962.39.925.

31. Etissa E, Dechassa N, Alamirew T, Alemayehu Y, Dessalegne L (2014) Response of fruit quality of tomato grown under varying inorganic N and P fertilizer rates under furrow irrigated and rainfed production conditions. International Journal of Development and Sustainability 3: 371-387.

32. Polat E, Demir H, Erler F (2010) Yield and quality criteria in organically and conventionally grown tomatoes in Turkey. Scientia Agricola (Piracicaba Brazil) 67: 424-429.

33. Thakur, B.R., Singh, R.K., Nelson, P.E., (1996). Quality attributes of processed tomato products: A review. Food Rev. Int. 12, 375–401.

34. Tareen, M.J., Abbasi, N.A., Hafiz, I.A., (2012). Effect of oxalic acid treatments on storage life of peach fruit Cv. " Flordaking". Pak. J. Bot. 44, 207–310.

35. Jeong JH, Lee OM, Jeon YD, Kim JD, Lee NR, Lee CY, Son HJ (2010) Production of keratinolytic enzyme by a newly isolated feather degrading *Stenotro-*

*phomonas maltophilia*that produces plant groth promoting activity. Process Biochem 45:1738–1745. https ://doi.org/10.1016/j. procb io.2010.07.020.

36. Tiquia SM (2002) Evaluation of organic matter and nutrient composition of partially decomposed and composed and composted spent pig litter. Environ Technol 24:97–107.

37. Mc William D (2003) Identifying nutrient deficiencies for efficient plant growth and water use. Co-operative extensive services college of agriculture and home economics.

38. Zheljazkov DV (2005) Assessment of wood waste and hair waste as a soil amendment and nutrient source. J Environ Qual 34:2310– 2317. https ://doi.org/10.2134/ jeq20 04.0332.

39. Ghehsareh AM, Kalbasi M (2012) Effect of addition of organic and inorganic combinations to soil on growing property of greenhouse cucumber. African Journal of Biotechnology 11: 9102-9107.

40. Mehta, N., Asati, B.S., (2008). Genetic relationship of growth and development traits with fruit yield in tomato (Lycopersicon esculentum Mill). Karnataka J. Agric. Sci. 21, 92–96.

41. Parbha KP, Loretta YL, Usha RK (2007) An experimental study of vermin-biowaste composting for agricultural soil improvement. Bioresource Technology 99: 1672-1681.

42. Ilupeju EA, Akanbi WB, Olaniyi JO, Lawa BA, Ojo MA, Akintokun PO (2015) Impact of organic and inorganic fertilizers on growth, fruit yield, nutritional and lycopene contents of three varieties of tomato (*Lycopersicon esculentum* (L.) Mill) in Ogbomoso, Nigeria. African Journal of Biotechnology 14: 2424-2433.

43. Wang XX, Zhao F, Zhang G, Zhang Y, Yang L (2017) Vermicompost improves tomato yield and quality and the biochemical properties of soils with different tomato planting history in a greenhouse study. Frontiers in Plant Scienc8:1978. doi: 10.3389/ fpls.2017.01978.

44. Dar, R.A., Sharma, J.P., (2011). Genetic variability studies of yield and quality traits in tomato (Solanum Lycopersicum L.). Int. J. Plant Breed. Genet. 5, 168–174.

45. Mendelova A, Flkselova M, Mendel L (2013) Carotenoids and Lycopene content in fresh and dried tomato juice. Acta Universitatis Agriculturae et SilviculturaeMendelianaeBrunensis 5:1329– 1337. https :// doi.org/10.11118 /actau n2013 61051 329.

46. Huang XY, Liu YW, Di DL, Liu JX, Li C (2010) An Improved LCDAD method for simultaneous determination of lutein, b-carotene and lycopene in tomato and its products. Chromatographic 71(¾):331–334. https ://doi.org/10.1365/s1033 7-009-1417- 0**(ISSN: 0009-5893).**

47. Somers, G.F., Kelly, W.C., Hamner, K.C., Somers, G.F., Kelly, W.C., Hamner, K.C., (1951). Influence of nitrate supply upon the ascorbic acid content of tomatoes. Am. J. Bot. 38, 472–475.

48. Subbiah, K., Perumal, R., 1990. Effect of calcium sources, concentration, stages and number of sprays on physicochemical properties of tomato fruits. South Indian Horti. 38, 20–27.

49. Dabire C, Sereme A, Parkouda C, Somda MK, Traore AS (2016) Influence of organic and mineral fertilizers on chemical and biochemical compounds content in tomato (*Solanum lycopersicum*) var. Mongal F1. Journal of Experimental Biology and Agri-

cultural Sciences 4: 631-636.

50. Eivazi AR, Rastegarni AR, Habibzadeh Y, Mogaddam A, Khlilzadeh G (2013) Influence of manure fertilizers on morpho-physiological traits of tomato (*Lycopersicunescutentum*Mill). Pakistan Journal of Agricultural Sciences 1: 89-93.

51. Dabire C, Sereme A, Parkouda C, Somda MK, Traore AS (2016) Influence of organic and mineral fertilizers on chemical and biochemical compounds content in tomato (*Solanum lycopersicum*) var. Mongal F1. Journal of Experimental Biology and Agricultural Sciences 4: 631-636.

52. Youssef MA, Eissa MA (2016) Comparison between organic and inorganic nutrition for tomato. Journal of Plant Nutrition. DOI: 10.1080/01904167. 2016.1270309.

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Isolation and Characterization of Protease Producing Novel *Burkholderia sp.*PS1 from Soil Sample and its Protease Production Optimization Studies

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Abstract

Extracellular proteases, due to their commercial, industrial applications have become significant and targeted for scientific research during current research. Our present report states about unfolding of potential proteases from soil sample. Even though there are diversified protease positive microorganisms was reported but still few are *Burkholderia Cenocepacia* proteases are unrevealed. Selective soil sample was collected from different places of Mahbubnagar Telangana state for screening and isolated potential protease producing microorganisms. 10^{-5} diluted sample spread over skim milk agar (High Media) and selected 108 protease positive organisms from screening. selected isolates are pushed to secondary screening, picked highest zone of hydrolysing microorganism was identified through 16s rRNA generated 1358bp amplicon was forwarded to NCBI blast, 98% homology *Burkholderia Cenocepacia* (Bks) submitted and generated accession number MH290479 with optimized Bks protease production showing maximum production at temperature 30°C, P^H -7, Inoculum size 3%, 1% glucose as carbon source,0.5% Gelatine protein and Zn metal ion was recorded.

Key words: *Burkholderia* sp, Bks, Optimization, Proteases, *Bacillus* Sp. NCBI Blast, Production.

Introduction

Microbial enzymes are core components of bio-industrial processes and they are cheaper, reliable, high yielding diversified biocatalyst. Proteases are one of the leading commercially important biocatalysts in enzymatic pool. Microbial proteases are extensively used in pharmaceutical, detergent, dairy, baking, leather and biomedical engineering for formulation of drugs, cleaning, making of edible products and reducing environmental pollution.

These enzymes profound applications in feather processes in poultry, detergent industries, pharmaceuticals, biotransformation, food processing, silk gumming, bioremediation, biosynthesis of drugs and bioconversions (1-4). The most common protease producing organisms are gram positive *Bacillus* sp. comparatively less in other species (3). Identification and screening research was active since first protease from Bacillus licheniformis was industrially commercialized as an additive in detergents in the 1960s (5) Numerous *Bacillus*-derived proteases have been isolated, purified and characterized their optimization studies to know efficiency of protease activity. Uplift of protease depends on abiotic stability with broad substrate specificity in short period of fermentation, easy downstream processing, and less cost have been

implicated (6). Many of the Bacillus-derived proteases are elevated exposure to temperatures and pH, but the majority proteases are incompatible with detergent matrices (2,5).

Apart from bacillus sp. and Pseudomonas sp. Fewer investigations was observed in protease field. Burkholderia sp. is also one of the potential protease producing gram negative bacteria it also shown efficient production observed under abiotic stress *Burkholderia cepacia, Burkholderia stabilis*. (7). Hence, proteases with optimization require for superior performance for industrial exploitations, especially for commercial utility are being sought. Although colourable screening and identification of most of the enzymes have already been found in *Bacillus thermantarcticus*, and *Bacillus mojavensis* etc. (1,8). Since investigations are continuous to find novel, efficient, ease on adoptable microbial proteases been targeted in future studies. In the present study, isolation of unexplored industrially efficient protease producing organisms from soil samples and its optimization studies relevance to commercial purposes.

Materials and Methods

Collection of soil sample

Different Soil sample was collected from various places in Mahbubnagar town and different places of Telangana state specially cattle dung landfills, poultry forms dump yard and undisturbed soils was taken around10-15cm depth keeping natural habitat without any disturb.

Primary screening

Autoclaved sterile water used for Serial dilution and 10^5,10^6,10^7 and 10^8 dilutions was taken for spread plate method done for collected soil on screened 1% skim milk agar and incubated it for 36hrs on 37^0C the bacterial culture which shown clear zones around the colonies was selected for progressive studies. The zone of proteolytic efficiency was determined by measuring the diameter of the colony and zone of hydrolysis. Efficient bacterial isolates *Burkholderia Cenocepacia*, Bacillus cereus, Staphylococcus aureus and Bacillus subtilis like Bacteria showed good proteolytic activity Such colonies were selected and sub cultured on nutrient agar slants and preserved at 4^0C for further study.

Assay for protease activity by plate method

To Identify effective protease producing bacteria from pre-screened, isolated positives AP1- Bacillus Sp., AP6-*Burkholderia Cenocepacia*, AP7-Bacillus cereus AP8- Staphylococcus Sp., was streaked skim milk agar (HiMedia) and left for 48hr incubated plates shown zone of hydrolysis.

Proteolytic activity

Most common substrate Azocasein (HiMedia) taken as substrate for defining the Protease catalytic activity of *Brukholderia sp* PS (9). 2ml Eppendorf reaction was prepared mixture contains 120 µL of enzyme extract and followed by added 480 µL of 10 mg /ml of 0.2M and 7.2P^H azocasein solution. Later mixture was kept under incubated for 1hr at 37 °C and later reaction was stopped by adding 600µl trichloroacetic acid (TCA) 30% (w/v). After centrifugation at 10,000 g for 5 min, 800 µl top of the later mixed with 200 µl 1·8M NaOH. The absorbance at 440 nm was measured in a UV -spectrophotometer (9). Unit for enzyme was defined as the amount that caused an increase of 0·01 in absorbance at 420 nm in the assay conditions (10).

Biochemical and molecular identification

Readymade gram staining kit was used for morphological identification performed (HiMedia grams), DNA was extracted using the genomic DNA isolation kit (HiMedia) was used a (Mo-Bio-Laboratories) and meanwhile 16S rRNA sequencing sequenced as described previously (11). Taq polymerase supplied by Bio-Rad was used for PCR, which was started

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with the primers 16S-F 5' GTTTGATCCTGGCTCAG-3' and 16SR5'-AAGGAGGTGATCCAGCCGCA-3'. The resultant partial sequence of the 16S rRNA was obtained and this sequence was performed NCBI Nucleotide BLAST to NCBI Nucleotide BLAST was showing similarity of 98.93% *Burkholderia Cenocepacia* contained 1358bp nucleotides. The molecular identificatio16S rRNA sequencing was performed at Bioserve india pvt, and identified *Burkholderia cenocenocepician*, Accesion number- MH290479. nearest taxa was with construction of Phylogenetic trees was constructed using phylogenetic tree-making algorithms, consensus sequence maximum-likelihood (ML) was selected and noted similar sequences generated on of using the PhyML program (12) and neighbour-joining (NJ) (13) using the PHYLIP package, version 3.5 (14), and the resultant tree topologies were evaluated by bootstrap analysis based on 1000 resampling. Using the SEQBOOT and CONSENSE programs in the PHYLIP package. Pairwise evolutionary distances were evaluated and computed on DNADIST program with the Kimura 2-parameter model was developed (15).

Production of protease from Burkholderia Cenocepacia

Highly efficient protease *Burkholderia Cenocepacia* colony from skim milk agar plate was inoculated in 100ml mineral medium NaCl -0·5g, K_2HPO_4-0·3g, KH_2PO_4-0.6g, $MgSo_4$ -0.1g containing 1%g casein/100ml prepared and adjusted to pH 7·0. The production of protease was placed on orbital shaker in 250 ml flasks containing 100 ml of the medium by incubation for 72 h at 37 °C. The culture was centrifuged at 10,000 g for 10 min and the supernatant was used as crude enzyme.

Optimization of protease production

Effect of inoculums size

Inoculum size increased continuous sets 1%,2%, 3%,4% and 5% Inoculum size utilise the available components that enhance the protease production. Maximum protease production was recorded with 3% inoculum size with *Brukholderia cenocepcia*.

Optimization of temperature and pH protease production

To find maximum production of protease various abiotic factors was adopted temperature and pH on the proteolytic activity were determined. Thermal stability was evaluated by pre incubation of the enzyme for up to 20 min at 40, 45 and 50°C then residual activity was measured as described above. The assay for optimum pH was developed using 0·1 mol/l (pH 6, 7, 8 and 9), or sodium carbonate buffer (pH 9 and 10).

Effect of carbon and nitrogen sources

Major carbon sources like glucose, Maltose, Sucrose, Lactose fructose starch was tried with 1% w/v and Nitrogen substrates like casein, soybean extract, yeast extract, beef extract, Peptone, Ammonium nitrate and potassium nitrate 0.5% of w/v was taken for protease production and thereafter 1 ml of inoculum was inoculated into the medium and then subjected to submerged showed maximum enzyme production with glucose and beef extract was noted.

Effect of metal ions

Different essential metals like Mn^{+2}, Ca^{+2}, Mg^{+2}, Zn^{+2}, Fe^{+2} of 10mM concentrations was applied for media optimization. significant change was observed in the production of protease with Ca^{+2} on *Burkholderia Cenocepacia* and observed in 2-fold *by Ca and Zn* increased production.

Result and Discussion

Screening and isolation of potential protease producing bacteria

Mixture of soil sample dilutions spreading on skim milk agar generated 108 positive

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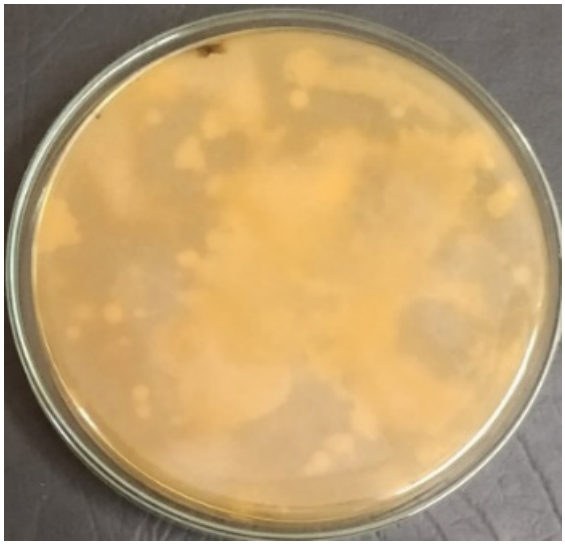

Fig.1 Primary screening on skim milk agar

Fig.2 Selective screening on skim milk agar, AP1- Bacillus subtilis, AP6-Burkholderia Cenocepacia, AP7-Bacillus cereus AP8- Staphylococcus aureus

bacterial as a result of from primary screening shown in figure.1. Selective culture showing maximum zone of hydrolysis clearly indicating that AP6 *Burkholderia sp. PS1* (Bkc-AP6) showing maximum zone hydrolysis compared to other organisms, Positive outcomes boosted to work on *Burkholderia sp.PS1* protease production and intent to know optimization of production media for futuristic industrial applications.

Fig.3 Secondary screening on skim milk agar AP6-Burkholderia Cenocepacia

Biochemical charecterization

Gram negative rod shaped structures are visualised undermicroscopic examination,resuted table.1 Cells are, motile colonies on NA (Nutrinet agar) are circular, 2 mm in diameter, White, crateri form and entire. Cells grow from 18 to 35 C with an optimum temperature of 37 ^{0} C and growth occurs in a pH range of 7 to 10 was analysed. More over biochemical charecterisation was conduted result was shown in Table .1 Catalase, oxidase, gelatinase,protease, indole and urease are positive, but vogeus proskeur , methyle red , lipase,bitrilase reductase are negative. nitrate reduction, H_2S production, Voges–Proskauer reaction and methyl red reaction are negative similarity to Burkholderia sp.

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Table.1.Biochemical identification of effeciant protease producing orgonism

S. No.	Name of the test	PS1
1	Gram's staining	Negative
2	Shape	Rods
3	Motility	Motile
4	Indole test	Positive
5	VP reaction	Negative
6	MR reaction	Negative
7	iso-Citrate test	Positive
8	Catalase	Positive
9	Oxidase	Positive
10	Phosphatase	Positive
11	Lipase	Negative
12	Urease	Positive
13	Gelatinase	variable
14	Protease	Positive
15	Nitrate reduction	Negative
16	H_2S production	Negative

The partial sequece of reulsted amplified product streamed to NCBI, blast search engine generated similar queries of 1358bp nucleotides sequence was showing 100% query coverage with Zero E value result made confrimation that species belongs to Burkholderia sp.nov.PS1 was confirmed. Further submitted 1358bp sequence and generated Accention number –MH290479 named as Burkholderia cenocepicia.

Construction of phylogenitic tree

On the basis nucleotide sequence generated 1358 subjected to phylogenetic relationship of strain PS1-AP6 with previously reported species in BLAST sequence similarity search (NCBI-BLAST/EzTaxon). The results indicated that at the 16S rRNA gene sequence level, strain PS1 -AP6 was close to the phylogenetic adjacent are with 98.97% similarity *Burkholderia cenocepecia* GP3 MN240932 and Burkholderia sp. MaAL 241, (Accesion number KY810687) for1358bp with 100% blast query. Phylogenetic tree based on ML (Fig. 4). and NJ (Fig.4) trees further indicated that strain PS1-AP6 clustered with Burkholderia cenocepecia GP3 MN240932 and Burkholderia sp. MaAL 241with a phylogenetic distance of 98.97% each and distinct from the other species of the genus. Despite the high 16S rRNA gene sequence similarity. Above blast and phylogenetic analysis indicating that strain *Burkholderia sp.nov.* PS1 is could be assigned to a novel species.

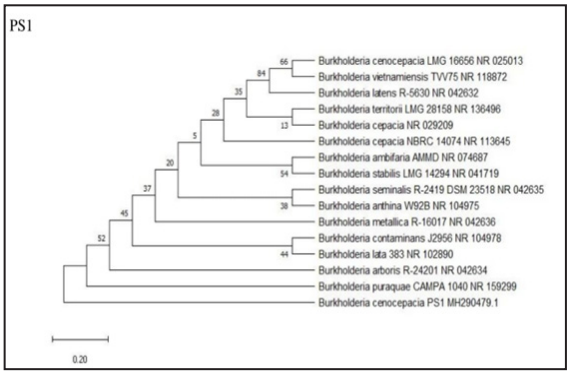

Fig.4 Phylogenitic tree constructed for *Burkholderia sp*.nov.PS1 (AC.No- MH290479)

Optimization of protease production

Globally commercialised proteases require not only isolation and identification but also industrial level utility like processeing, production and mass yeilding of an enzyme, present study optimization of production culture media revealed data of Bks protease production through submerged flask based experiment relavance to industrial applications. Size of an inoculam plays crusial role find where orgonism is enterning stationary phase, In inculum size versus production of enzyme clearly showing fig.5 inciating that maximum production has poitnted in 185 U/mL/Min at 3% later increasing inoculum also no significant difference was observed.

For Enhance mass production of enzymes, abiotic physical and chemical factors optimization is mandatory. Diffent ranges of P^H fig .7 from 4-10 and fig. 6 temperature 20-50^{o}C observations are recoreded Maximum production of Bks proease at P^H-7 (173 U/mL/Min), 35^{o}C –(165U/mL/Min) it give confirmation that Bks protease is neutral optimum nearest to

room temperature are suitable. Critical suistainability of orgnisms depends availability of carbon, nitrogen sources. where as in Bks protease production. 1% W/v of Glucose in production media 176 U/mL/Min of enzyme is produced. Compare to other carbon sources like starch (polysacharides) and disacharides (lactose, Sucrose) are less effect as carbon source than monosacharieds(Glucose Fructose,galctose) in another context Gelatine and casein like nitrogen sources leads enzymes production but ammonium nitrate as well as potasium nitrates are no significant effect was noted.

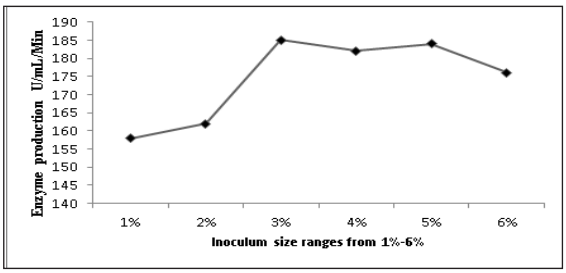

Fig.5 Effect of inoculum size on protease production

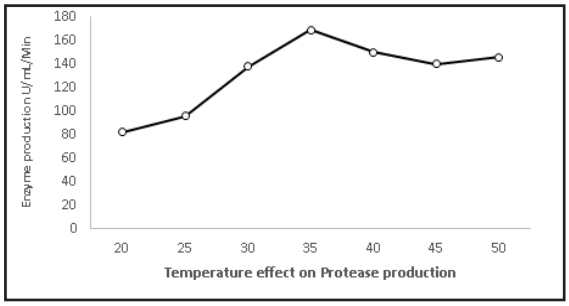

Fig.6 various temperature effect on protease production

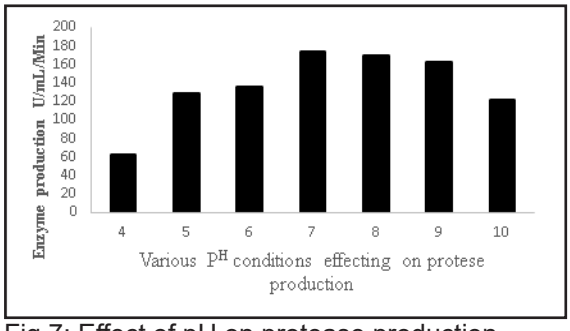

Fig.7: Effect of pH on protease production

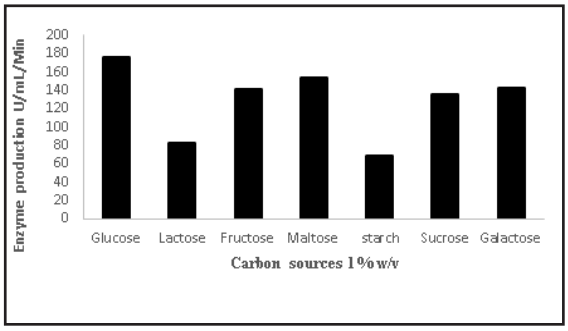

Fig.8 Effect of carbon source with 1% w/v size on protease production

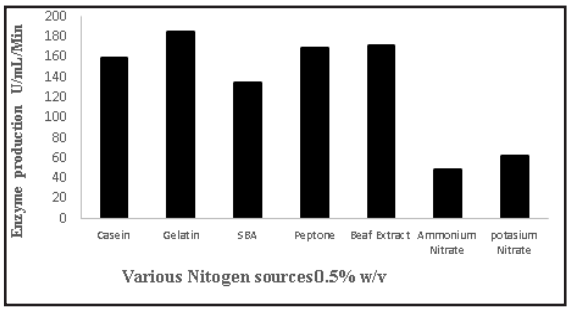

Fig.9 Effect Nitrogen sources on protease production

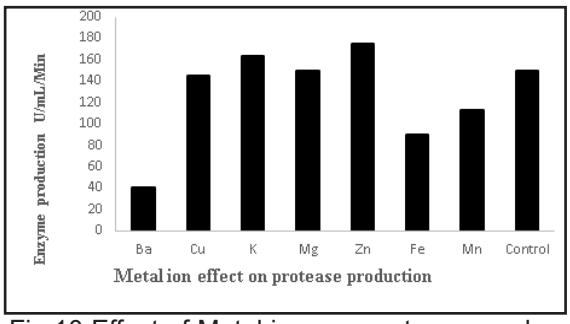

Fig.10 Effect of Metal ions on protease production

Conclusion

Isolation identification of Efficient Proteases are always plays crucial role in industrial enzymatic pool, our present study, Protease positive *Burkholderia Cenocepacia* was isolated from soil was optimised protease production temperature30oC, PH7, 3% w/v inoculum 1%v/v glucose and 0.5% gelatine is more suitable condition to produce protease. Highest

protease was recorded as 185 U/mL/Min under presence of Gelatine, 3% inoculum and metal ions like Zink and calcium. As Bks protease has more applications in industry, hence protease production from *Bks* is recommended.

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References

1. N. Bhaskar, E.S. Sudeepa , H.N. Rashmi , A. Tamil Selvi (2007) "Partial purification and characterization of protease of Bacillus proteolyticus CFR3001 isolated from fish processing waste and its antibacterial activities. "Bioresource technology" Volume 98, Issue 14.

2. Gupta, R., Beg, Q.K., Lorenz, P. (2002) Bacterial alkaline proteases: molecular approaches and industrial applications. "Appl. Microbiol. Biotechnol". 59: 15 32.

3. Jellouli K, Bougatef A, Manni L, Agrebi R, Siala R, Younes I, Nasri M (2009). Molecular and biochemical characterization of an extracellular serine-protease from Vibrio etschnikovii J1. "J. Ind. Microbiol. Biotechnol". 36: 939-948.

4. Sareen, R., Mishra, P. (2008). Purification and characterization of organic solvent stable protease from Bacillus licheniformis RSP-09 37. "Appl. Microbiol. Biotechnol". 79: 399.

5. Katsuhisa Saeki, Katsuya Ozaki, Tohru Kobayashi (2007) Susumu Its Detergent alkaline proteases: enzymatic properties, genes, and crystal structures, "Journal of biosciences and bioengineering". June;103(6)

6. Maurer, K. Detergent proteases. Current Opinion in Biotechnology, (2004) 15, 330-334.

7. Maria de Fátima Barros Leal Nörnberg, Marilene Lenz Mentges, Silvana Terra Silveira, Eduardo César Tondo and Adriano Brandelli (2011). A psychrotrophic Burkholderia cepacia strain isolated from refrigerated raw milk showing proteolytic activity and adhesion to stainless steel. Journal of Dairy Research 78 257–262.

8. Dipasquale L., Calandrelli V., Romano I., Nicolaus B., Gambacorta A., Lama L. (2008). Purification and characterisation of a highly thermostable extracellular protease from Bacillus thermantarcticus, strain M1. "Ann. Microbiol". 58 (2) 253-259.

9. Yewande Suberu a b, Idowu Akande a, Titilola Samuel a, Adekunle Lawal b, Ademola Olaniran (2003). Cloning, expression, purification and characterisation of serine alkaline protease from Bacillus subtilis RD7. "Biocatalysis and Agricultural Biotechnology" Volume 20, July 2019, 101264.Syst Biol . Oct;52(5):696-704.

10. R.C.S. Thys, F.S. Lucas, A. Riffel, P. Heeb, A. Brandelli (2004). Characterization of a protease of a feather-degrading Microbacterium species Get access Arrow. Letters in Applied Microbiology, Volume 39, Issue 2, 1 August 2004, Pages 181–186.

11. Lane, D. J. (1991). 16S/23S rRNA sequencing. In Nucleic Acid Techniques in Bacterial Systematics, pp. 115–175. Edited by E. Stackebrandt & M. Goodfellow. Chichester: Wiley.

12. Stéphane Guindon, Olivier Gascuel (2003) A simple, fast, and accurate algorithm to estimate large phylogenies by maximum likelihood. Syst Bi. 2003 Oct;52(5):696-704.

13. Saitou, N. & Nei, M. (1987). The neighbor-joining method: a new method for reconstructing phylogenetic trees. Mol Biol Evol 4, 406–425.

14. Felsenstein, J. (1993). PHYLIP (phylogeny inference package). 3.5.1. University of Washington, Seattle, USA: Department of Genome Sciences.

15. . Kimura, M. (1980). A simple method for estimating evolutionary rates of base substitutions through comparative studies of nucleotide sequences. J Mol Evol 16, 111–120.

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Enhanced Catecholamine Production in *Gomphrena globosa* Suspension Cultures

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Abstract

Secondary metabolites (SMs) are small organic molecules produced by plants that are mostly not essential for their growth, development and reproduction but are produced to confer a selective advantage to the plant. Since their concentration in the plant in a natural environment are low, the plants are many a times stimulated to enhance their production especially when they have significant commercial and medicinal applications. Elicitation is a technique used in tissue culture wherein external elicitors, either biotic or abiotic, induce stress in the plant thereby leading to stimulation of production of specific plant secondary metabolites. Catecholamines and their precursors have many medicinal applications and have been reported to occur in *Gomphrena* sp. The present study successfully identified elicitors (chitosan and copper sulphate) for the enhanced production of catecholamines and its precursors from suspension cultures of *G. globosa*. Both the biotic and abiotic elicitors were used in different concentration so as to compare the effect of each on catecholamine and/or precursor concentrations in the suspension cultures. Successful elicitation of these precursors as well as catecholamines was standardized using *G. globosa* callus as a model system.

Keywords: Catecholamines, *Gomphrena globosa,* LCMS/MS, elicitation, suspension culture

Introduction

Plant secondary metabolites include a wide class of chemicals that have commercial applications as antibiotics, flavoring agents, fragrances, insecticides, dyes etc. Many of them also have medicinal applications and are therefore economically desirable. These secondary metabolites are not necessary for the survival of individual cells of the plants but rather are involved in interaction of the cell with its surrounding thereby ensuring their survival. In the natural environment, production of these secondary metabolites amounts to less than 1 % of the dry weight of the plant and is highly dependent on the developmental stage of the plant (1, 2). Since they are production is on an on-demand basis, commercial production of these useful metabolites from plants grown in their natural habitats would not be economical.

In such a scenario, plant tissue culture becomes a valuable tool in mass production of these commercially valuable metabolites which could be enhanced by controlled additions of elicitors (both biotic and abiotic). Research also suggested that faster proliferation rate and biosynthetic cycle in cell and organ culture leads to higher metabolic rate and consequently increased synthesis of valuable secondary metabolites when compared with field grown plants (3). Stress in any form is a crucial factor in altering the phytochemical profile of the plant. Elici-

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tation using external elicitors will induce stress in the plant thereby leading to stimulation of production of specific plant secondary metabolites. Elicitors are chemically distinct from each other and various factors like type of elicitor, concentration, duration of exposure, treatment schedule, culture type, cell line, medium composition, presence or absence of growth regulator, and age or stage of the culture at the time of elicitor treatment are the major factors that can determine the effectiveness of the elicitation strategies on biomass and secondary metabolite production (4 -9).

Research suggested that abiotic elicitors like nitrogen source, sucrose and phosphate in the medium affect the concentration of L-DOPA in tissue culture of *Mucuna pruriens* (10). Scientists have also reported several fold increase in concentration of L- DOPA in presence of biotic elicitors like methyl jasmonate, pectin, chitin or precursor tyrosine in suspension cultures of *M. pruriens* and *M. prurita* (11). Production of dopamine has also been reported in the cell suspension cultures of *Celosia argentea* var. *plumosa* (12). Previous work has proved the presence of catecholamines and predecessors in the inflorescence of *Gomphrena globosa* (13). The present study attempted to induce callus production from *G. globosa*, quantify the catecholamines present in the callus and then the use of elicitors (chitosan as the biotic elicitor and copper sulphate as abiotic elicitor) to increase the production of catecholamines viz. dopamine and epinephrine as well as catecholamine precursors viz L-tyrosine and L-DOPA.

Materials and Methods

Callus induction

Plant material for callus induction

The plant *G. globosa* was grown in the garden of Ramnarain Ruia Autonomous College, Mumbai. Node, internode and leaf of *G. globosa* were selected as explants for induction of callus. These surface sterilized explants were then separately transferred under aseptic conditions within a laminar air flow to Murashiga and Skoog (MS) basal medium supplemented with BA (1 mg/L) + NA (0.1 mg/L) and MS basal medium with 2,4 D (1 mg/L) + Kn (0.5 mg/L). The tubes inoculated with the explants were kept in a culture room for callus initiation at 25 ± 2ºC, 9 hr photoperiod under white fluorescent light (3000 lux). The hormonal combination that showed the best callusing was selected for subculturing and elicitation studies.

Proliferation and Maintenance of the callus

For callus maintenance, one month old calli were transferred to MS + 2,4 D (1 mg/L) + Kn (0.5 mg/L). The cultures were maintained at 25 ± 2°C under a 9 hr photoperiod at 3000lux light intensity. After sufficient quantity of the callus was obtained, they were subjected to elicitation studies.

Elicitation study

Establishment of callus suspension culture

The callus of *G. globosa* was maintained on MS+ 2,4 D (1 mg/L) + Kn (0.5 mg/L). The cell suspension culture was established in liquid MS medium supplemented with the same hormones. This suspension culture was used for elicitation studies using chitosan (100 mg/L and 200 mg/L) as the biotic and copper sulphate (0.1mg/L and 0.5 mg/L) as the abiotic elicitors.

0.5 gm of *G. globosa* callus was transferred in 40 ml of the liquid MS medium containing 2,4 D (1 mg/L) and Kn (0.5 mg/L).

The flasks were kept on shaker incubator (at temperature between 25 ± 2°C at an rpm between 70-80) for a period of 5 days followed by addition of the elicitors and then subsequently removing the culture bottles on Day 5, Day 10 and Day 15 respectively.

Sample preparation

The suspended cells (after incubation) were weighed and then subjected to methanolic extraction for 18 hrs. The filtrate was also subjected to extraction of catecholamines and

precursors in methanol in the same manner. The callus extracts and the filtrate extracts were then used for estimation of catecholamines by LCMS/MS.

Standard preparation

10 ml of the 1000 ppm stock solution of the standards; L-Tyrosine, L-DOPA, Dopamine and Epinephrine; were prepared. The standards were dissolved in minimum quantity 0.1 N HCl and the volume made upto 10 ml with methanol.

LC parameters

HPLC (Shimadzu Prominence Binary Gradient System, Shimadzu Corporation, Japan) equipped with a binary pump (20AD), degasser, an autosampler (SIL-20AC), a temperature-controlled column compartment CTO-20AC and photodiode array detector (SPD-M20A) was used. Chromatographic data was acquired using Labsolutions software. The analysis was done using Shim-pack MAqC-ODS I (150 mm x 4.6mm I.D.,5µm) column. The mobile phase comprised of-(A) 0.1% formic in water (B) 0.1% Formic acid in acetonitrile in a gradient mode.

Results and Discussion

Of the hormonal combinations used best callusing was observed for leaf explant of *G. globosa* on MS+2,4-D (1 mg/L) + Kn (0.5 mg/L). Previous work has reported the presence of catecholamines and predecessors in the inflorescence of *G. globosa* (13). Light dependent synthesis of epinephrine, norepinephrine and dopamine in *Portulaca grandiflora* callus has been reported by researchers (14). An attempt was therefore made in the current study to detect the catecholamines and precursors by stimulating their synthesis through elicitation using biotic and abiotic elicitors.

LCMS/MS quantitation of L-tyrosine, L-DOPA, dopamine and epinephrine from both callus and filtrate was carried out. On considering the total concentration (from callus and filtrate) of L-tyrosine was highest on day 10 (3.898±0.191 ppb) with 200 mg/L chitosan while the total concentration of L-DOPA was highest on day 15 (0.785±0.075 ppb) when 0.5% $CuSO_4$ was used (Fig 1 and 4). The total concentration of dopamine was highest on day 15 (0.689±0.053 ppb) when 0.5% copper sulphate was used as the elicitor whereas, the total concentration of epinephrine was highest on day 10 (3.258±0.630 ppb) with 200 mg/L chitosan (Fig 2 and 3). The current study presented that 200mg/L chitosan led to a four-fold increase of L-tyrosine(precursor) on day 10 from callus and a 3.3-fold increase in epinephrine on day 10. Similar high concentration of epinephrine was reported in callus of *P. grandiflora* (14). The current study also observed that with 0.5% $CuSO_4$ a seventy-fold increase in L-DOPA concentration on day 15 and a fifty-two-fold increase in dopamine concentration on day 10 was observed. A 9.25-fold increase in L-DOPA content in the callus of *H. enneaspermus* and presence of dopamine (not more than 42.08 mg/ gm dry weight) in suspension cultures of *Celosia argentea* var. *plumosa* has been reported by researchers (12, 15). In general, significant accumulation of catecholamine precursor L-tyrosine was observed in callus when chitosan was used as elicitor while L-DOPA accumulated when copper sulphate was the elicitor. Dopamine and epinephrine concentration were lower than control callus concentrations. Quantitation of the catecholamines and their precursors from filtrate showed high concentration of L- tyrosine and epinephrine by day 10 when chitosan was the elicitor while L-DOPA and dopamine production enhanced by day 15 with copper sulphate as elicitor. The present work also concluded that of all the components quantitated, the content of L-tyrosine was the highest followed by epinephrine, L-DOPA and dopamine respectively. The study also noted that while L- tyrosine was maximally retained in the cell, L-DOPA, dopamine and epinephrine were maximally released into the medium. L-tyrosine, a precursor of L-DOPA and catecholamines (dopamine and epinephrine), is an amino acid required for protein synthesis as well as for synthesis of other commercially important secondary metabolites

like betalains, capsaicin, benzophenanthridine, rosmarinic acid, coumaric acid, caffeic acid, cinnamic acid, securinine, phenylethanoid glycosides, other alkaloids and phenolics to name a few (16- 24). The maximal retention of L-tyrosine within the cell and its high content can therefore be attributed to it being an important precursor of protein synthesis and betalains which are known to be produced in *G.globosa*.

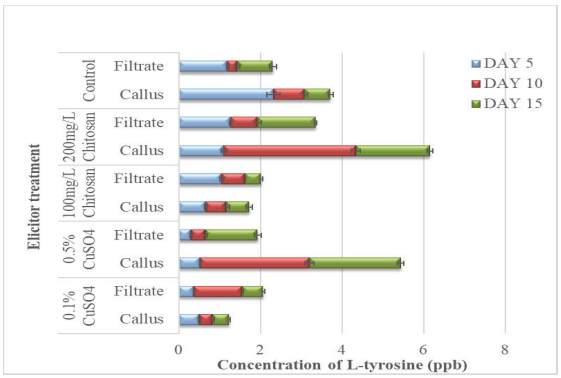

Fig.1: Quantitation of L-tyrosine(ppb) from suspension culture of *G.globosa* in presence of biotic and abiotic elicitors.

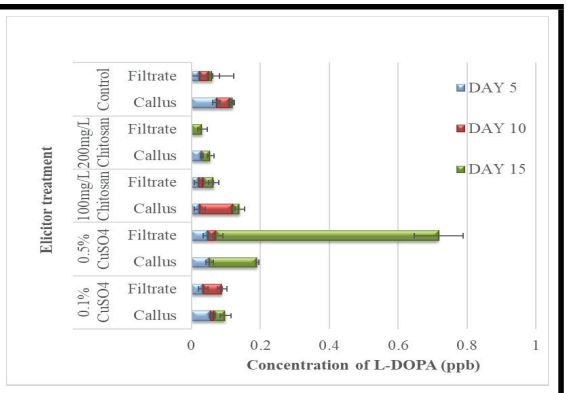

Fig.2: Quantitation of L-DOPA (ppb) from suspension culture of *G.globosa* in presence of biotic and abiotic elicitors.

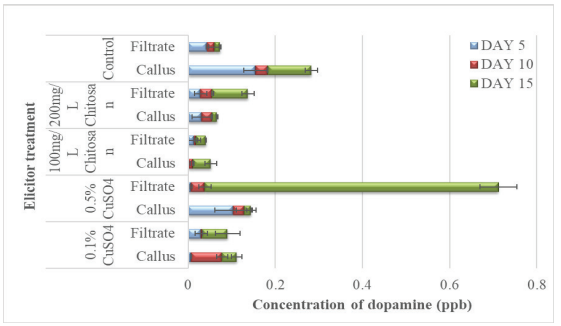

Fig.3: Quantitation of dopamine (ppb) from suspension culture of *G.globosa* in presence of biotic and abiotic elicitors.

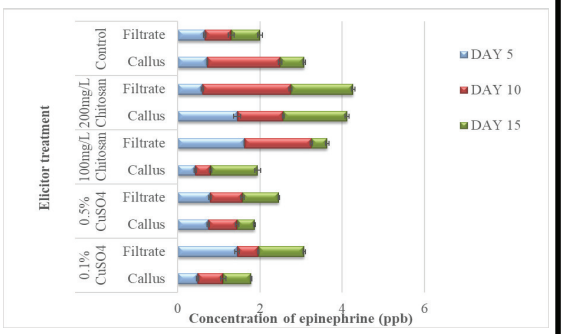

Fig.4: Quantitation of epinephrine (ppb) from suspension culture of *G.globosa* in presence of biotic and abiotic elicitors.

Conclusion

The LCMS/MS quantitation showed that chitosan was the best elicitor for enhancing L-tyrosine and epinephrine content while copper sulphate was best suited for enhancing L-DOPA and dopamine concentrations. The present study can be used as a model system to increase production these metabolites from other plant sources via tissue culturing.

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References

1. Dixon, R.A. (2001). Natural products and plant disease resistance. *Nature,* 411**:** 843–847.

2. Oksman-Caldentey, K. M., and Inzé, D. (2004). Plant cell factories in the post-genomic era: new ways to produce designer secondary metabolites. *Trends in Plant Science*, **9(9):** 433-440.

3. Rao, R.S. and Ravishankar, G.A. (2002). Plant cell cultures: chemical factories of secondary metabolites. *Biotechnology Advances*, 20**:**101–153.

4. Namdeo A. (2007). Plant cell elicitation for production of secondary metabolites: a review. *Pharmacognosy Reviews,* 1**:** 69–79.

5. Ramakrishna, A. and Ravishankar, G. A.(2011). Influence of abiotic stress signals on secondary metabolites in plants. *Plant Signaling and Behavior*, 6**:** 1720–1731.

6. Wang, J. W. and Wu, J. Y.(2013). Effective elicitors and process strategies for enhancement of secondary metabolite production in hairy root cultures. *Biotechnology of Hairy Root Systems,* 55–89.

7. Naik, P. M. and Al–Khayri, J. M.(2016). Abiotic and biotic elicitors–role in secondary metabolites production through *in vitro* culture of medicinal plant. *Abiotic and Biotic Stress in Plants-Recent Advances and Future Perspectives*, 247–277.

8. Dhiman, N., Patial, V., and Bhattacharya, A. (2018). The current status and future applications of hairy root cultures. In *Biotechnological Approaches for Medicinal and Aromatic Plants* (pp. 87-155). Springer, Singapore.

9. Kaur.K. and Pati. P. K. (2018). Stress-induced metabolite production utilizing plant hairy roots. *Hairy Roots- An Effective Tool of Plant Biotechnology*, 123–145.

10. Wichers, H.J., Visser, J.F., Huizing, H.J. and Pras, N. (1993). Occurrence of L-DOPA and dopamine in plants and cell cultures of *Mucuna pruriens* and effects of 2, 4-D and NaCl on these compounds. *Plant Cell, Tissue and Organ Culture*, **33:**259–264.

11. Raghavendra, S., Kumar, V., Ramesh, C.K. and Moinuddin Khan, M.H. (2012). Enhanced production of L-DOPA in cell cultures of *Mucuna pruriens* L. and *Mucuna prurita* H. *Natural Product Research*, 26(9)**:** 792-801.

12. Guadarrama-Flores, B., Rodriguez-Monroy, M., Cruz-Sosa, F., Garcia-Carmona, F., and Gandia-Herrero, F. (2015). Production of dihydroxylated betalains and dopamine in cell suspension cultures of *Celosia argentea* var. *plumosa*. *Journal of Agricultural and Food Chemistry*, 63(10)**:** 2741-2749.

13. Sandhya M, Jessy P and Shailesh D: LCMS/MS Quantitation of catecholamines and their predecessors from *Portulaca oleracea* and *Gomphrena globosa International Journal of Pharmaceutical Sciences and Research,* 12(11): 1000-06.

14. Endress, R., Jager, A. and Kreis, W.,

(1984). Catecholamine Biosynthesis Dependent on the Dark in Betacyanin-forming *Portulaca* callus. *Journal of Plant Physiology,* **115:** 291- 295.

15. Sathish, S., Vasudevan, V., Karthik, S. , Pavan, G. and Manickavasagam, M. (2019). Enhanced L-DOPA production from elicited cell suspension culture of *Hybanthus enneaspermus* (L.) F. Muell. *Plant Biotechnology Reports,* **13:** 613–621.

16. Parry, R.J. (1978). Biosynthesis of the Securinega alkaloids. Investigations of the early and late stages of the pathway. *Bioorganic Chemistry,* **7(3):** 277-288.

17. Zenk, M.H. (1994). The formation of benzophenanthridine alkaloids. *Pure and Applied Chemistry*, **66(10-11):** 2023-2028.

18. Veeresham,C., Kokate, C.K., Venkateswarlu, V. and Ramesh Babu, B. (1994). Influence of DLDOPA and L- tyrosine on biproduction of capsaicin in callus culture of *Capsicum annum* Linn. *Indian Journal of Experimental Biology*, 32: 223-224.

19. Croteau, R., Kutchan, T. M., and Lewis, N. G. (2000). Natural products (secondary metabolites). *Biochemistry and Molecular Biology of Plants*, 24: 1250-1319.

20. Ouyang,J., Wang,X., Zhao, B. and Wang, Y.(2005). Enhanced production of phenylethanoid glycosides by precursor feeding to cell culture of *Cistanche deserticola*. *Process Biochemistry,* 40(11)**:** 3480-3484.

21. Namdeo, A. G., Jadhav, T. S., Rai, P. K., Gavali, S., and Mahadik, K. R. (2007). Phcog Rev.: Review Article Precursor feeding for enhanced production of Secondary metabolites: A review. *Pharmacognosy reviews*, 1(2):227-231.

22. Roy, D., and Mukhopadhyay, S. (2012). Enhanced rosmarinic acid production in cultured plants of two species of *Mentha*. *Indian Journal of Experimental Biology*, **50:** 817-825.

23. Martins, N., Roriz, C. L., Morales, P., Barros, L., and Ferreira, I. C. F. R. (2017). Coloring attributes of betalains: A key emphasis on stability and future applications. *Food and Function*, 8(4)**:** 19–23.

24. Wang, M., Lopez-Nieves, S., Goldman, I. L., and Maeda, H. A. (2017). Limited tyrosine utilization explains lower betalain contents in yellow than in red table beet genotypes. *Journal of Agricultural and Food Chemistry*, 65(21)**:** 4305-4313.

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An *In-Silico* Approach towards Drug Discovery against Bacterial DNA Gyrase using Nalidixic Acid

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Abstract

Escherichia coli is a gram- negative bacteria and is common for bacterial disease that affects the intestinal tract. DNA gyrase A was an enzyme which is responsible for DNA breakage and reunion are mostly released by *Escherichia coli* bacteria in human gastrointestinal tract, causing a situation called gastroenteritis. Normally in native DNA gyrase A protein, the 83th position is occupied by serine residue which is mutated to phenylalanine making the enzyme as antibiotic resistant against the known antibacterial drugs. Nalidixic acid is mainly used in the treatment for urinary tract infections caused by gram-negative bacteria. Here, according to our present study, we compared binding affinities of native protein structure and mutated protein structure of *E. coli* DNA gyrase A against the Nalidixic acid. ADMET properties, bioactive scores and other parameters of the ligand structure were calculated using various tools including SwissADME, pkCSM software as well as PreADMET web tool. Lastly molecular docking study was carried out using AutoDock Vina software and the results were evaluated on the basis of iMod server. After comparing the docking scores, it was observed that the mutated protein (-5.5 kCal/mol.) shows more binding affinity towards nalidixic acid than the native protein (-5.3 kCal/mol.) Although, more *in-vitro* and *in-vivo* studies need to be performed to get a satisfactory conclusion.

Keywords: Bacterial DNA Gyrase, Drug discovery, Nalidixic acid, Molecular docking interaction.

Introduction

DNA gyrase is an enzyme which belongs to class of Topoisomerase enzyme; more specifically Topoisomerase II. This enzyme mainly catalyses the ATP-dependent negative super-coiling of double-stranded closed-circular DNA (1,2). It is divided into two subunits preferentially known as Gyrase A and Gyrase B; among which DNA Gyrase A (GyrA) is made up of two functional groups like N-terminal and C-terminal. DNA-protein bridges which are formed by N-terminal responsible for breaking and re-joining function whereas C-terminal was responsible for DNA-binding non-specifically (3-5). GyrA was used for DNA cleavage and ligation for the tyrosine which is an active site of GyrA. This enzyme is resistant to multiple fluoroquinolones in enteric bacteria because of the spontaneous mutation happens in GyrA enzyme. *'Escherichia coli releases the DNA Gyrase A enzyme in human gastrointestinal tract that may not be directly related to gastroenteritis.* As per the literature study it has been noted that mutation occurs at 83rd position of GyrA making the bacterial genome as antibiotic resistant. One more such variation occurs where serine at 83rd position was mutated to phenylalanine (S83F). "Sandhya Bansal; Vibha Tandon. (2011). Contribution of mutations in DNA gyrase

and topoisomerase IV genes to ciprofloxacin resistance in Escherichia coli clinical isolates. , 37(3), 253–255"

In case of drug discovery for such disorder, we need to have mutated GyrA structure which is not available in the database. So, this sequence structure gap in the study will be very useful for understanding the drug discovery approach (6, 7). In account of drug discovery Nalidixic acid ($C_{12}H_{12}N_2O_3$) is such a synthetic quinolone and antibacterial agent that can be active against mostly gram-negative organisms (8-11). Due to mutation in the GyrA sequence, it has become antibiotic resistance which destabilizes the structure upon binding. "Pourahmad Jaktaji R, Mohiti E. Study of Mutations in the DNA gyrase gyrA Gene of Escherichia coli. Iran J Pharm Res. 2010 Winter;9(1):43-8" To find the protein-ligand interaction stability between the native and the mutant structure by using sequence analysis and molecular docking, we have taken Nalidixic acid as the ligand, which could show the stability result upon binding with our desired protein (Native and Mutant) structure so that we could determine that weather Nalidixic acid has any positive role against the disease gastroenteritis or not (Fig 1). The direct and rational process for drug discovery was molecular docking and here the protein-ligand binding energies calculated in kilo calories per mole (Kcal/mol).

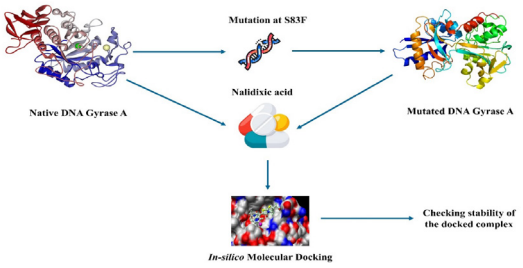


Fig. 1: The Graphical Abstract

Materials and Methods

Selection and preparation of Receptor or Protein

For the purpose of protein selection, 6RKU protein has been selected. This protein denotes the crystal structure of *E. coli* DNA Gyrase A subunit, belonging to ligase family. From Protein Databank (http://www.rcsb.org/), the 3D structure of 6RKU (Fig 2) has been obtained. Then to stabilize the receptor structures, the already attached ligands and water molecules were removed by BIOVIA Discovery Studio 2020 software (https://discover.3ds.com/discovery-studio-visualizer-download/) (12). In order to prepare the mutant version of the protein structure, SPDBV software (https://spdbv.unil.ch/) has been used. By using this software, the serine residue at 83rd position of the native protein was mutated to phenylalanine.

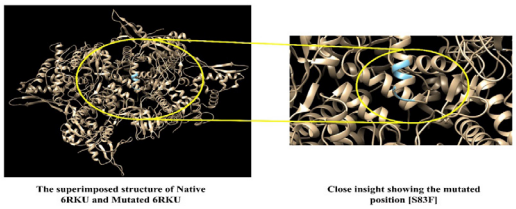


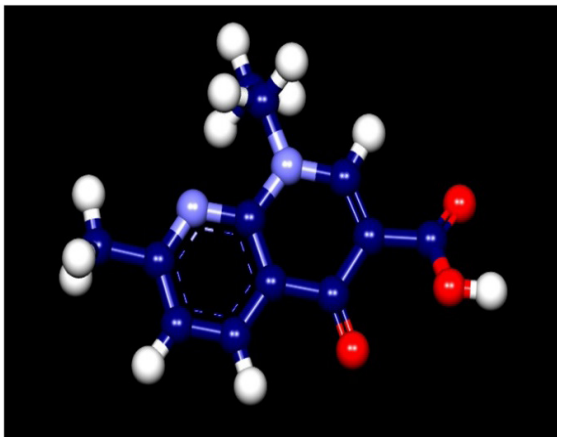
Fig. 2: The Superimposed Structure of Both the Native 6RKU and Mutated 6RKU

Validation of protein structure

The newly generated protein PDB structure was then undergone through a series of quality analyses including ERRAT, Procheck using SAVES 6.0 (https://saves.mbi.ucla.edu/) (13, 14), and ProSA-web (https://prosa.services.came.sbg.ac.at/prosa.php/) (15).

Selection and preparation of the ligands

Chemical compounds, or more precisely, bioactive ligands i.e., Nalidixic acid, was chosen on the basis of literature study (Fig 3). 2D Structure Data Format (SDF) of all chemical compounds or ligand files were retrieved from PubChem (www.pubchem.ncbi.nlm.nih.gov/) and later translated to their 3D PDB format using Open Babel software. A PDBQT format file was created after adding (16) hydrogenating atoms and the desired torsion to a PDB format



file.

Fig. 3: The 3D Chemical Structure of Nalidixic acid

Validation of compound structures

SwissADME prediction of the compounds

Adsorption, Distribution, Metabolism and Excretion all-together termed as ADME is a very profitable process to assess all those previously mentioned parameters of the ligands using the server of SwissADME website (https://www.swissadme.ch/) (17, 18).

Toxicity prediction of the compounds

In case of drug designing and establishment of a suitable drug compound, it is a very necessary step to predict the toxicity level of the small compounds or rather ligands before investigating their endurance capacity when ingested into any animal model like mouse, rat as well as in human too. There are two online servers available for these purposes, they are: PreADMET server (https://preadmet.bmdrc.kr/) (19, 20) and pkCSM (Predicting Small-Molecule Pharmacokinetic Properties Using Graph-Based Signature) (http://biosig.unimelb.edu.au/pkcsm/) (21). In case of PreADMET server, first the SDF structures retrieved from the “Pourahmad Jaktaji R, Mohiti E. Study of Mutations in the DNA gyrase gyrA Gene of Escherichia coli. Iran J Pharm Res. 2010 Winter;9(1):43-8” Pubchem were converted to mol2 format using Open babel software and then submitted to the online server for toxicity prediction: mutagenicity (AMES test), carcinogenicity (for rat and mouse) and hERG inhibition. On the other hand, SMILEs structure of each ligand, derived from Pubchem database were directly submitted to the pkCSM server to analyse the results of LD50 (mol/kg) and highest permissible dosage for human (log mg/kg/day).

Molecular docking interaction using AutoDock Vina

AutoDock Vina software (http://vina.scripps.edu/) (22) for molecular docking and virtual screening that significantly improves efficient binding mode predictions, thereafter gives more accuracy in protein-ligand interaction. AutoDock Vina works by calculating the grid maps and clusters. Before proceeding to final docking step, Kollman charges and other modifications were added to the purified form of protein and converted into a proper readable PDBQT file format. Similarly, ligand is also transformed into PDBQT file. A grid box on active residues of protein was generated with different grid dimensions and centres but with similar spacing i.e., 0.375. The exhaustiveness was set at 8 and binding energy affinity was predicted with AutoDock Vina software. The final visualization of docked structure was performed using BIOVIA Discovery Studio 2020 (https://discover.3ds.com/discovery-studio-visualizer-download/) and PYMOL software (https://pymol.org/) (23).

Assessment of structural hotspots and binding pockets on the receptor protein

An online server called CASTp 3.0 (http://sts.bioe.uic.edu/) (24) is used to predict active amino acid residues, or alternatively structural hotspots on the receptor protein. A systematic quantitative characterisation of the surface topography of proteins is often provided by the Computer Atlas Surface Topography of Protein (CASTp).

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Results and Discussion

Validation of protein structure

According to the predictions made by the aforementioned web tools, Fig 4 depicts the overall quality of recognition of the 3D protein PDB structure native 6RKU as well as mutant 6RKU. The process of approving an ideal protein structure involves confirming the protein PDB model using a number of quality control metrics. According to ERRAT's results, both the native and mutant the proteins exhibit a quality score of 85%, indicating that the protein is well-modelled. The "overall quality factor" for non-bonded atomic interactions is displayed, with higher values denoting higher quality. Moving on, the ProSA-web result displayed the protein's total z score. In this case, the scores are -7.95 (native) and -7.88 (mutant), indicating that both the structures are located within the X-ray region. The Ramachandran plot of the protein models then showed that, in accordance with the PROCHECK result, 86% of residues were found in the most preferred regions, followed by 13.0% in additional allowed and 0 % in generously allowed and disallowed regions. The collective outcomes derived from the previously mentioned attributes indicate that native and mutant version of 6RKU protein is of high quality and appropriate for additional molecular interaction analysis.

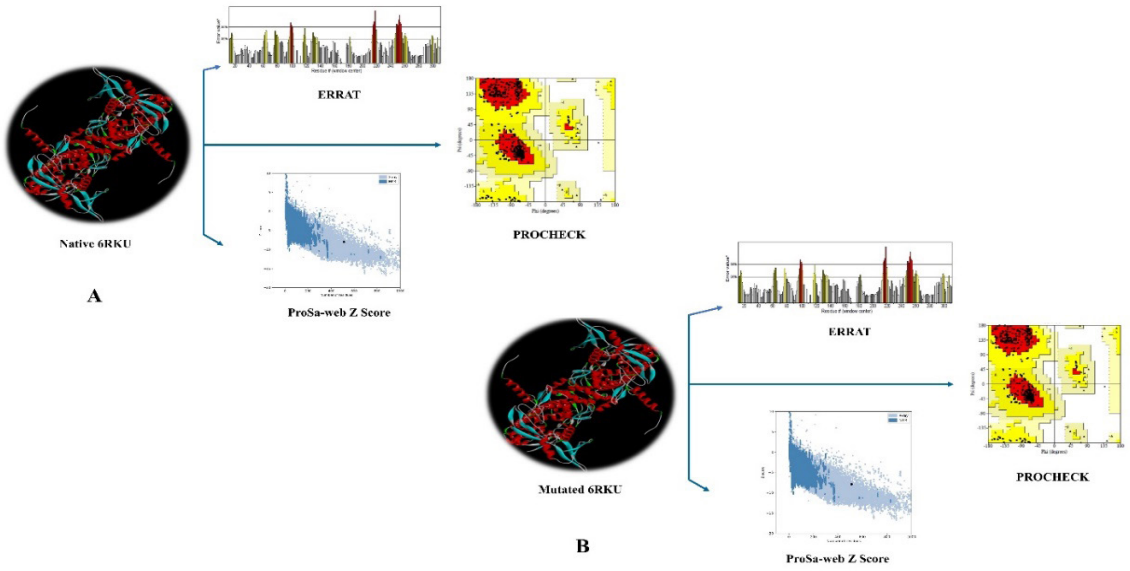

Fig. 4: Results Showing Quality Checking Parameters of [A] Native 6RKU and [B] Mutated 6RKU

Validation of compound structures

SwissADME prediction of the compounds

It is intended for the study to apply a variety of *in-silico* techniques to assess the computational aspects of the therapeutically active elements. Upon submission of ligand structure in SMILEs format, SwissADME results are generated based on ADME/toxicity analysis and Lipinski filter analysis upon submission of the ligand structure in SMILEs format. Here in our result, we have represented the outcome of the drug likeliness data in a tabulated manner. (Table 1)

According to the SwissADME server derived drug likeliness result, from Table 1, Nalidixic acid shows satisfactory result with 0 violations.That means, nalidixic acid follow Lipinski's rule 5 completely (Molecular weight must not exceed 500 Dalton; Hydrogen bond donors and

acceptors must not exceed 5 and 10 respectively and last but not least, the octanol-water partition coefficient should not cross 5) (25). In addition, bioavailability scores describe how quickly and how much a molecule enters the bloodstream after oral delivery, eventually reaching the desired areas, according to Table 1, all selected compounds show the similar score i.e., 0.85. This value implies that the compounds have 85% probability of being bioavailable (26).

Table 1: Showing Results of Drug Likeliness of Nalidixic acid

Name of the Compound	Lipinski's Rule		
	Satisfactory	No. of Violations	Bioavailability Score
Nalidixic acid	Yes	0	0.85

Toxicity prediction of the compounds

Assessing the toxicity of small compounds is an essential stage in the drug discovery approach. The PreADMET server's toxicological prediction result, which includes the chemicals' hERG inhibition, mutagenicity, and carcinogenicity are shown in Table 2. According to the result, the negative prediction translates carcinogenic activity whereas positive means the compound possess no carcinogenic activity (18). Talking about the mutagenic characteristics, all compounds are mutagenic in nature. In case of hERG inhibition, most of the compounds show medium to low probabilities of blogging hERG gene that often associated with sudden heart attacks in humans (27). The result obtained from pkCSM server is given in Table 3.

The term "LD50" refers to the concentration of a test substance deemed to be lethal for 50% of the test subjects in the treated group; in this case, the lethal dose for rats has been determined and is stated in terms of mol/kg. It is one method of determining a compound's acute toxicity, or short-term poisoning potential.

The highest dose or quantity of a medicine or test substance that does not manifest any undesirable side effects is referred to as the maximum tolerated dose. It is identified through multiple clinical trials that involve gradually raising the dosage in various human populations until and unless a tolerable adverse impact is observed. The unit of expression is log mg/kg/day (28).

Table 2: Showing Results of Toxicity Analysis of Nalidixic acid

Name of the compound	Toxicity			
	Mutagenicity	Carcinogenicity		
		Rat	Mouse	hERG inhibition
Nalidixic acid	Mutagen	Negative	Negative	Low risk

Table 3: Showing Results of LD 50 Value and Maximum Tolerated Dose for Human of Nalidixic acid

Name of the Compound	Oral Rat Acute Toxicity (LD 50)	Maximum Tolerated Dose (Human)
Nalidixic acid	2.48	1.644

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Molecular docking interaction using AutoDock Vina

The binding affinity of nalidixic acid with the necessary protein crystal structure of E. coli DNA Gyrase A subunit (6RKU, 6RKU_m) is ascertained based on the docking analysis performed by AutoDock Vina. The ligand with the highest binding affinity to the proteins is the one with greater negative binding energy. We have chosen nalidixic acid based on our research, and it exhibits a unique set of results due to variations in its binding capacity with the target protein receptors (6RKU, 6RKU_m). As reported by the docking result, nalidixic acid shows the maximum binding affinity i.e., -5.5 kcal/ mol. with 6RKU_m followed by -5.3 kcal/mol. against 6RKU. Replace this line with Fig 5A-5B show the 3D and 2D interactions mode between the protein and the ligand molecule.

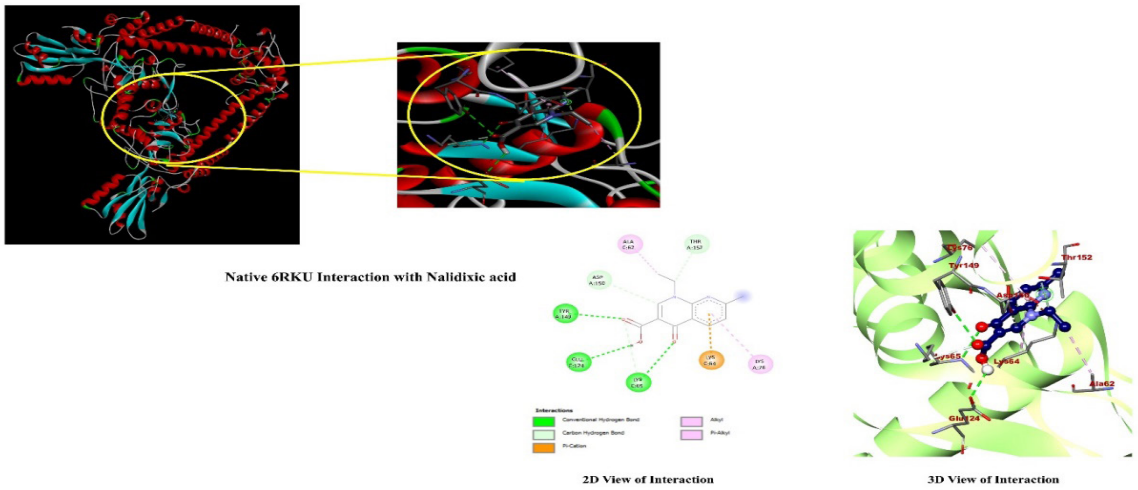

Fig. 5A: Figure Showing Molecular Docking Interaction Between Native 6RKU And Nalidixic Acid with Respective 2D and 3D View of Interactions

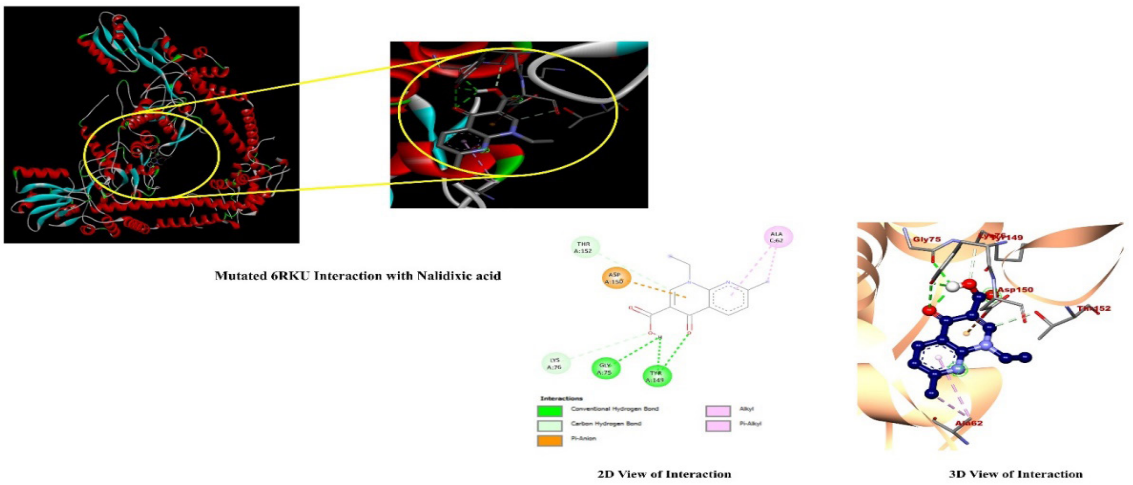

Fig. 5B: Figure Showing Molecular Docking Interaction Between Mutated 6RKU And Nalidixic Acid with Respective 2D and 3D View of Interactions

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Assessment of structural hotspots and binding pockets on the receptor protein

Table 4 displays the findings for the 3D protein PDB structure 6RKU and 6RKU_m. obtained from the CASTp 3.0 online server. This outcome demonstrates the key amino acids involved in the particular protein-ligand interaction.

Table 4: Table Showing Active Amino Acid Residues Obtained via Molecular Docking Interaction for Each of the Receptor Proteins [6RKU and 6RKU_m] Along with the Ligand

Name of the compound	Name of the protein	Active Amino Acid Residues
Nalidixic acid	6RKU	ALA 62, GLY 75, LYS 76, TYR 149, ASP 150, THR 152
	6RKU_m	ALA 62, LYS 64, LYS 65, LYS 76, GLU 124, TYR 149, ASP 150, THR 152

Fig 6 shows the active binding pockets which are present in the 3D protein (6RKU). Each binding pockets represent the ligand attachment region on the protein. Binding pockets (1-5) are differentiated on the basis of their size and volume.

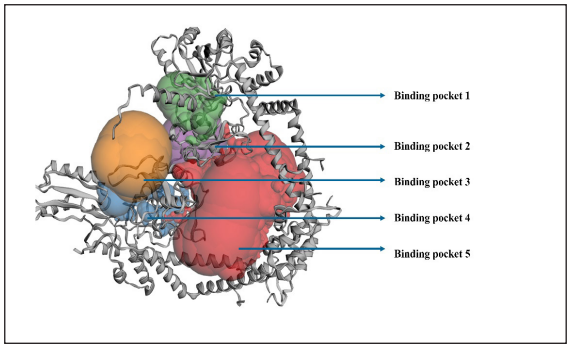

Fig. 6: Figure Showing the Active Binding Pockets on The Protein 6RKU

Conclusion

Virtual screening via molecular docking is one of the most popular approaches to evaluate the binding possibility between a receptor protein and ligand structures. Generally, molecular interaction study shows that protein-ligand binding only happens when free energy change is negative along with increasing negative value of the binding energy corresponds the increased stability between protein-ligand complex. According to our study, we have performed docking interactions between the native and mutant form of bacterial DNA gyrase with a common quinolone antibiotic Nalidixic acid. Upon carrying out and alongside validating all the necessary variables for *in-silico* screening, it was revealed that nalidixic acid showed more stable interaction with the mutant version of bacterial DNA gyrase than the native one. From this initial virtual work, we can conclude that nalidixic acid showed active target towards the E. coli DNAs, by exhibiting positive interaction against mutated version of the target protein. Furthermore, to establish this hypothesis into original theory, more *in-vivo* and *in-vitro* studies are required.

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Conflict of Interest

There are no conflicts of interest, the authors declare.

References

1. Frédéric Collin 1, Shantanu Karkare, Anthony Maxwell. Exploiting bacterial DNA gyrase as a drug target: current state and perspectives. Appl Microbiol Biotechnol. 92(3):479-97 (2011).

2. Angehrn P, Buchmann S, Funk C, Goetschi E, Gmuender H, Hebeisen, P, Kostrewa D, Link H, Luebbers T, Masciadri R, Nielsen

J, Reindl P, Ricklin F, Schmitt-Hoffmann A, Theil FP. New antibacterial agents derived from the DNA gyrase inhibitor cyclothialidine. J Med Chem 47(6):1487–1513 (2004).

3. Ashiuchi M, Kuwana E, Yamamoto T, Komatsu K, Soda K, Misono H. Glutamate racemase is an endogenous DNA gyrase inhibitor. J Biol Chem 277(42):39070–39073 (2002).

4. Ashiuchi M, Kuwana E, Komatsu K, Soda K, Misono H. Differences in effects on DNA gyrase activity between two glutamate racemases of Bacillus subtilis, the poly-gammaglutamate synthesis-linking Glr enzyme and the YrpC (MurI) isozyme. FEMS Microbiol Lett 223(2):221–225 (2003).

5. Baker NM, Weigand S, Maar-Mathias S, Mondragon A. Solution structures of DNA-bound gyrase. Nucleic Acids Res. 39(2):755–766 (2011).

6. Bradbury BJ, Pucci MJ. Recent advances in bacterial topoisomerase inhibitors. Curr Opin Pharmacol 8(5):574–581 (2008).

7. Champoux JJ. DNA topoisomerases: structure, function, and mechanism. Annu Rev Biochem 70:369–413 (2001).

8. Bauernfeind, A., and G. Grtimmer. Biochemical effects of nalidixic acid on Escherichia coli. Chemotherapia 10:95-102 (1965).

9. Bourquignon, G. J., M. Levitt, and R. Sternglanz. Studies on the mechanism of action of nalidixic acid. Antimicrob. Agents Chemother. 4:479-486 (1973).

10. Javor, G. T. Inhibition of ribonucleic acid synthesis by nalidixic acid in Escherichia coli. J. Bacteriol. 120:282-286 (1974).

11. Pedrini, A. M., D. Geraldi, A. Siccardi, and A. Falaschi. Studies on the mode of action of nalidixic acid. Eur. J. Biochem. 25:359-365 (1972).

12. BIOVIA, Dassault Systèmes, [Discovery Studio Client], (2020).

13. Colovos C, Yeates TO. Verification of protein structures: patterns of nonbonded atomic interactions. Protein Science 2(9): 1511-1519 (193).

14. De Oliveira CCS, Coutinho Pereira GR, De Alcantara JYS, Antunes D, Caffarena ER, De Mesquita JF. In silico analysis of the V66M variant of human BDNF in psychiatric disorders: An approach to precision medicine. PLoS One 14(4): e0215508 (2019)

15. Wiederstein M, Sippl MJ. ProSA-web: interactive web service for the recognition of errors in three-dimensional structures of proteins. Nucleic Acids Research 35: W407-W410 (2007).

16. Rezaei-Seresht H, Cheshomi H, Falanji F, Movahedi-Motlagh F, Hashemian M, Mireskandari E. Cytotoxic activity of caffeic acid and gallic acid against MCF-7 human breast cancer cells: An in silico and in vitro study, Avicenna journal of phytomedicine 9(6): 574–586 (2019).

17. Daina A, Michielin O, Zoete V. SwissADME: a free web tool to evaluate pharmacokinetics, drug-likeness and medicinal chemistry friendliness of small molecules. Sci. Rep 7: 42717 (2017).

18. Mishra SS, Sharma CS, Singh HP, Pandiya H, Kumar N. In silico ADME, Bioactivity and Toxicity Parameters Calculation of Some Selected Anti- Tubercular Drugs. Int J Pharm Phytopharm Res 6(6):77-79 (2016).

19. Cunha EL, Santos CF, Braga FS, Costa JS, Silva RC, Favacho HA, Hage- Melim LI, Carvalho JC, da Silva CH, Santos CB. Computational investigation of antifungal compounds using molecular modeling and prediction of ADME/ Tox properties. Journal of Computational and Theoretical Nanoscience 12(10):3682-91 (2015).

20. Bruce NA, Gurney EG, James AM, Bartsch H. Carcinogens as Frameshift Mutagens: Metabolites and Derivatives of 2-acetylaminofluorene and other Aromatic Amine Carcinogens. PNAS 69(11): 3128–3213 (1973).

21. Douglas EVP, Tom L. Blundell TL, Ascher DB. pkCSM: Predicting Small-Molecule Pharmacokinetic and Toxicity Properties Using Graph-Based Signatures. J. Med. Chem 58(9): 4066–4072 (2015).

22. Trott O, Olson AJ. AutoDock Vina: improving the speed and accuracy of docking with a new scoring function, efficient optimization and multithreading, Journal of Computational Chemistry 31(2):455-461 (2010).

23. Rehan M, Shafiullah. Medicinal plant-based saponins targeting COVID-19 Mpro in silico, Tradit Med Res 24 (2021).

24. Binkowski TA, Naghibzadeh S, Liang J. CASTp: Computed Atlas of Surface Topography of proteins. Nucleic Acids Res 31(13): 3352–3355 (2003).

25. Lipinski CA, Lombardo F, Dominy BW, Feeney PJ. Experimental and computational approaches to estimate solubility and permeability in drug discovery and development settings. Adv Drug Delv Rev 46(1-3):3-26 (1997).

26. Veber DF, Johnson SR, Cheng HY, Smith BR, Ward KW, Kopple KD. Molecular properties that influence the oral bioavailability of drug candidates. J Med Chem 45(12):2615-23 (2002).

27. Yu HB, Zou BY, Wang XL, Li M. Investigation of miscellaneous hERG inhibition in large diverse compound collection using automated patch-clamp assay. Acta Pharmacol Sin 37:111-23 (2016).

28. Singh S, Gupta AK, Verma A. Molecular properties and bioactivity score of the Aloe vera antioxidant compounds-in order to lead finding. Res J Pharm Biol Chem Sci. 4:876-81 (2013).

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Analyzing the Immunomodulatory Effects of Tea Poly-Phenols and in management of COVID-19

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Abstract

This study primarily aims to co-relate the health benefits of tea intake, an accessory dietary supplement as a preventive remedy against COVID-19 and the inclusion of derivatized polyphenols in addition to the alcohol based anti-viral disinfectants providing increased longevity. A methodical exploration of databases such as ScienceDirect, Taylor & Francis Online, and Google Scholar was conducted using a variety of keyword combinations. The abstracts of all the retrieved results were scrutinized, and any studies deemed irrelevant were excluded from further assessment. Tea Polyphenols have been known to contain several defensive phytochemical compounds namely catechins, theaflavins, tannins and flavonoids. Amongst these, catechins more particularly, Epigallocatechin gallate (EGCG), a major component of green tea along with its lipophilic derivatives and theaflavins from black tea account for additional biological significance. Recent literature and molecular simulation studies have reported their potency as anti-viral agents with their effectiveness against the SARS-CoV-2, commonly known as the novel coronavirus, causing global pandemic with several deaths worldwide. Further, we emphasize on the need to explore polyphenolic compounds as adjuvants with the approved class of drugs employed for combating the severe acute respiratory syndrome (SARS) virus. This would aid in developing a novel class of drugs with plant-based compounds adding to the cumulative effect against the viral infections. Although, EGCG has been studied extensively, in vitro, and in vivo clinical studies with respect to its anti-viral capability would necessarily provide a new horizon to the scientific and medical fraternity in formulating drugs with a higher ratio of natural to the synthetic counterparts, thereby decreasing toxicity.

Keywords

Covid-19, viral infections, tea polyphenols, EGCG, theaflavins

Introduction

The World Health Organization recently announced the spread of the epidemic disease and named it as the 2019-novel coronavirus disease (2019-nCoV also known as COVID19) (1). Coronaviruses are positive sense single standard enveloped RNA viruses that are known to spread out among mammals including humans and birds. They show spherical or slight pleomorphic structure when they are viewed under an electron microscope with a genome size of 26 – 36 kb. They have more than 20,000 nucleotides, it consists of pp1a and pp1ab, two polyproteins, and is broken down by viral proteases. Alpha, beta, gamma, and delta are the four kinds of coronaviruses that are distinguished phenotypically and serologically from one another. They are reported to cause respiratory, enteric,

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hepatic, and neurologic diseases(2). The main origin of the virus is speculated to be from a bat, which later becomes transmitted to humans by several other sources(3). The symptoms associated with this viral disease include majorly throat infection, cough, runny nose, fever and respiratory related issues(4). Ever since, several stringent preventive measures are being taken in order to curb the spread of the virus. The treatment strategies mainly involved include the use of antibiotics, corticosteroids, antiviral drugs, immunomodulatory drugs and oxygen therapy based on the extent of severity. Also, vaccines are being administered to safeguard individuals and to reduce the complications posed by the contraction of the SARS-CoV-2. However, it is imperative to include supplementary drug molecules as effective remedies to limit the transmission of the disease. Plant derived compounds serve to be efficient against a wide spectrum of diseases including the ones caused by viruses. Tea polyphenols, thus are being looked upon for their anti-viral properties. Literature studies envisage epigallocatechin-3-gallate (EGCG) to be one of the major constituents present in green tea, accounting for nearly 50% to 80% of a brewed cup of green tea(5). The counter partner to EGCG is the group of theaflavins found in black tea which are fermented or oxidized forms of green tea. Both of these components found majorly in green and black tea respectively, have garnered attention for their anti-viral potency. The inhibitory effects of EGCG on a wide array of viruses make it an alternative anti-viral agent. These compounds have been proven to be essential as additional dietary supplements. Thus, as a preventive measure against the prevailing viral disease are discovery of novel and effective drugs that can significantly reduce the load of virus in the body, and other options are development of multiepitope based vaccine for the control of virus infections (6). This review discusses about the myriad of health benefits gained on regular tea consumption acknowledging the previous research and literature based studies and puts forward an overview i) focusing mainly on the two value added components: EGCG and theaflavins ii) analyzing tea polyphenols as potential adjuvants and nutraceuticals agents providing defense against the novel corona virus iii) contributing to the future perspectives that would enable further research and development in synthesizing combinatorial drugs with a major proportion of naturally derived constituents against COVID-19 (Figure 1).

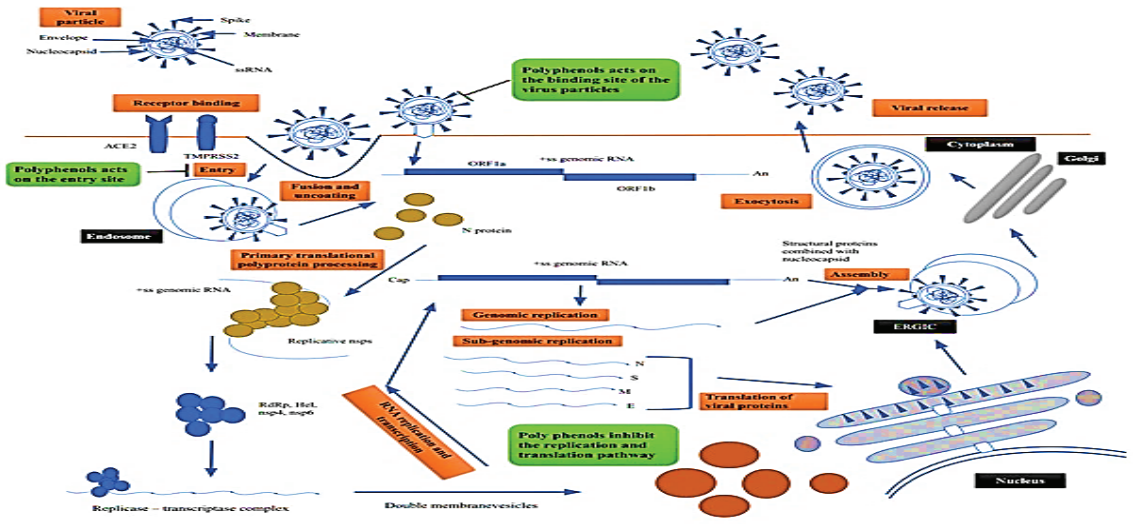

Figure 1. The SARS-CoV-2 virion and lifecycle depicting the application of polyphenols in battling COVID-19.

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Pathogenesis of Covid-19

The SARS-CoV-2 virus has a single strand and is positive RNA, with a size of around 29.9 kB. Research studies on these viral strains suggest them to be made up of four structural proteins, namely, the spike (S), membrane (M), envelop (E) and nucleocapsid (N) proteins (7) .The pulmonary epithelial cells specifically express the much-discussed receptor ACE-2 (angiotensin-converting enzyme-2), later identified to be the functional receptor for SARS-CoV. It has been speculated that this host receptor binds the S protein to initiate the host-pathogen interaction providing viral entry(8). According to research on these viruses, type II cells' microvilli and airway cells' apical cilia may be in charge of enabling viral entry (9). There are 14 open reading frames (ORFs) in SARS-CoV-2, and these ORFs encode for 27 distinct proteins (10). Replication and transcription of the viral genome is carried out using RNA-dependent RNA polymerase (RdRP/nsp12). Nsp7 and nsp8 are the associated co-factors required during the viral RNA synthesis catalyzed by RdRP(11). COVID-19 can be divided into the following three stages based on the extent of its infection:

Stage 1: Asymptomatic phase

This is the initial stage of infection occurring in the first two days upon the entry of SARS-CoV-2. The primary receptor involved here is ACE2. According to the in vitro studies, the conducting airways are where the ciliated cells first become infected. To fully comprehend their activity in vivo, more research is necessary. The innate response is limited at this stage. Nasal swabs are used for the detection of the viral infection. These are found to be more sensitive compared to the throat swabs providing effective results on analysis using RT-PCR.

Stage 2: Upper airway tract infection

The virus continues to spread and progress in the respiratory system, intensifying the body's defenses. Beta and lambda interferons are mostly produced by the virus-infected host epithelial cells (12). The disease is restricted to this stage for the majority of the patients and could be treated with home isolation with the prescribed medication. The pathogenicity of the virus, which in extreme cases is quite comparable to that of SARS, causes coronavirus to have a substantial unfavorable impact on the respiratory system. Most COVID-19 patients only have mild side effects; approximately 4% to 5% of severe cases require hospitalization or oxygen support. Severe cases of COVID-19 can result in septic shock, acute respiratory distress syndrome (ARDS), acute renal failure, heart failure, and sepsis. Age and chronic conditions have been identified as death risk factors. A high sequential organic failure assessment (SOFA) score has been verified in recent multivariate analyses of old age and has been linked to increased mortality.

Stage 3: Lower airway tract infection

Severity increases for some patients with the development of pulmonary infiltrates, and they enter stage 3 with the symptoms of hypoxia and progression to ARDS (Acute Respiratory Distress Syndrome). The virus now infects and multiplies within the alveolar type □ cells causing apoptosis and cell death (13). Eventually it leads to the destruction of both type □ and type □ alveolar cells. The end pathological result of SARS-CoV-2 is the severe alveolar damage. Due to their weakened immune systems and less capacity to heal injured epithelium, elderly individuals are more vulnerable. Most patients initially have a fever or no respiratory symptoms at all, but as lung tissue is lost, all patients eventually have varied degrees of pulmonary abnormalities. These conditions have been documented using lung scan imaging. Also, due to reduced mucociliary clearance in such patients, the virus readily is allowed to spread the gas exchange units of the lung.

Assessment of biochemical markers in Covid-19 patients

The timely management of Covid-19 makes the laboratory tests mandatory for controlling the viral spread and detection of the asymptomatic cases. Several serological tests are performed for monitoring the affected patients considering the associated biochemical markers. This leads to speedy isolation and in turn assists in containment of the disease (14). The laboratory parameters facilitate the understanding of the infection's severity while also predicting the likelihood that it will worsen and result in potentially fatal consequences like multiple organ failure (MOF), DIC, and ARDS (15). An elevated immune response called the cytokine storm causes damage to several tissues consequently worsening the disease progression (16). Higher blood concentrations of IFN-γ, IL6, IL10, and IL2 have been documented in severe cases. Analogously, in critically ill patients, neutrophil-to-lymphocyte ratio (NLR) and neutrophil-to-CD8+ T cell ratio (N8R) can be significant diagnostic variables. In addition, it has been noted that the serum levels of all cytokines (IL-2, IL-4, IL-10, IFN-γ, and TNF-α) except for IL-6 peak three to six days after the disease contraction (17). C-reactive protein, a plasma protein induced by different inflammatory mediators such as IL-6 is synthesized by the liver. It is used as a major clinical biomarker associated with inflammation (18). In addition to this, hepatic markers such as the alanine aminotransferase (ALT), AST and total bilirubin are routinely assessed in seriously ill patients indicating about the liver function (19). Additionally, cardiac, renal and pancreatic biomarkers are also assessed during the disease progression aiding in better treatment upon diagnosis. The elevated levels of C reactive proteins, lactate dehydrogenase (LDH), erythrocyte sedimentation rate (ESR), creatinine kinase, alanine aminotransferase (ALT), aspartate transaminase (AST), D-dimer, and low serum albumin reported in COVID-19 patients' serological diagnosis indicate sepsis, which may eventually lead to the development of multiorgan failure upon further progression.(20). This urges the need to safeguard we and develop a strong immune system that restricts the viral growth. Literature studies have entitled tea polyphenols to be potent immunity boosters.

Health benefits of regular tea intake

Tea polyphenols have gained considerable public attention since tea consumption is directly proportional to multiple health effects. In recent years, GTCs, especially EGCG, have been recognized to contain multi-purpose bioactive molecules contributing to antitumorigenic, anti-inflammatory, antioxidative, anti-proliferative, antibacterial, and antiviral effects. Green tea has been reported to possess antiviral activity toward coronaviruses(21). In accordance with a study conducted by Mandel, the inclusion of plant flavonoids in diet, for people with known family history would aid in a prophylactic treatment prior to contracting debilitating diseases such as AD or PD(22). Recent preclinical research suggests that the green tea component EGCG may be able to enhance the pharmacological effects of existing medications when used in a combination therapy regimen. Long term green tea intake leads to decreased oxidative stress with an increase in GSH/GSSG ratio and total SOD activity. However, it decreases protein oxidation, lipid peroxidation and modulates the CREB activation. This in a cascade eventually causes prevention of age-related spatial memory decline. Furthermore, tea polyphenols are expected to find application in the food industries as food preservatives due to their antioxidant and antimicrobial activities(23). Considering their anti-microbial ability, they are being explored lately as natural agents in fighting against several viral infections including COVID-19. Formulating valuable drug regulators to tackle the cytokine driven hyper inflammatory responses for the effective management of COVID-19 becomes essential. Despite the constant efforts of the scientific community, there occurs to be a dearth of effective drugs against the viral infections. In regard to

such circumstances, a new preventive antiviral approach with the application of tea polyphenols that turns out to be cost effective and pro environmental provides a fresh landscape in the medicinal domain. Polyphenols have been shown in earlier research to help reduce pro-inflammatory cytokines, such as kaempferol, resveratrol, epigallocatechin gallate, emodin, naringenin, apigenin, and curcumin, both in vitro and in vivo.(24, 25). Table 1 and 2 illustrate the numerous health benefits associated with the regular consumption of tea polyphenols and Table 3 highlights on the in vitro and molecular simulation studies with relevant viral molecular targets conducted recently.

Table 1. The in vitro studies evaluating anti-viral capabilities of polyphenolic compounds

Type	**Genome**	**Tea type Green/ black/ both**	**Compounds**	**Experimental Paradigm**	**Mechanism**	**Reference**
Coronavirus	ssRNA	Both	Theaflavins, EGCG	HRT-18	Neutralization Activity, molecular docking studies	(26)
Rotavirus	ssRNA	Both	Theaflavins, EGCG	BSC-1, Vero cells (ATCC CCL-81)	Neutralization Activity, anti-oxidative effect	(27)
Zika	ssRNA	Green	EGCG	Vero E6 cells	interaction of drug with the lipid envelope	(28)
Influenza A/H1N1	ssRNA	Green	EGCG	MDCK cells	Alteration in virus-host interaction	(29)
Influenza A and B	ssRNA	Black	theaflavins	MDCK cells	blockage of virus-receptor interaction and attenuation of viral replication	(30)
Dengue	ssRNA	Green	EGCG	Vero cells	causing deformation of the virus molecule	(22)
West Nile virus (WNV)	ssRNA	Green	EGCG	Vero cells (ATCC CCL-81)	Virucidal effect during the early stages of infection	(18)
Chikungunya	ssRNA	Green	EGCG	HEK 293T cells	Virus attachment inhibited	(16)
Ebola	ssRNA	Green	EGCG	HeLa cells, C57BL/6 mice	HSPA5 host factor was modulated	(31)
HIV-1	ssRNA	Both	EGCG and theaflavins	MT-2, H9/HIV-1	inhibits the entry of virus targeting gp41	(32)
HCV	ssRNA	Both	Theaflavins, EGCG	Huh-7 cells, Huh-7.5 cells	Inhibits virus- receptor interaction	(24)

Table 2. The in vitro and in vivo studies employing major polyphenolic compounds against chronic diseases based on previous research.

Cellular function	Polyphenol/ Compound	Experimental Paradigm	Mechanism	Reference
Anti-bacterial	GTP	C57BL/6 male mice	Increased CAT, SOD in the process of ileal injury caused by Salmonella typhimurium	(33)
Anti-fungal	EGCG	M. canis, T. mentagrophytes, T. rubrum	not clearly defined	(34)
Anti-aging	epicatechin	db/db mice	Improvement in the age-related biomarkers and modulates AMPK activity	(35)
Anti-obesity	Theaflavins EGCG	C57BL6 male mice	Promotes lipid metabolism by activating AMPK, ROS mediated blocking	(36)
Anti-oxidative	EGCG	Wistar male albino rats	Increased activities of CAT, SOD, GSR	(37)
Anti-inflammatory	EGCG	Spargue-Dawley rats (contusive SCI)	Regulated expression of TNF-α IL-1β, iNOS and COX-2	(38)
Anti-hypertensive	BTP and GTP	SHRSP rats	MLC phosphorylation lowered with anti-oxidative properties	(39)
Anti-proliferation	EGCG	MCF-7, H460, and H1975 cancer cells, H460 and MCF-7 mice models	Reduces IRS-1 levels and suppresses the MAPK pathway	(40)
Anti-cancer (Breast cancer)	EGCG	MCF-7 cells	Modulation of P53/Bcl-2 signaling pathway	(41)
Positive mood	Cocoa polyphenols	Volunteers	Involvement of the GABAergic systems	(42)
Anti-anxiety	Epicatechin	C57BL/6 mice	Elevates BDNF levels and modulates neurotrophic and monoaminergic signaling pathways	(43)
Anti-depressant	GTP	Male ICR mice	inhibits the hypothalamic–pituitary–adrenal axis	(44)
Anti-diabetic	L-EGCGd	STZ-induced diabetic rats	Inhibition of α-glucosidase	(45)
Anti-atherosclerotic	EGCG	ApoE$^{-/-}$ mice (C57BL/6J)	Regulation of LXR/SREBP-1 pathway	(46)
Cytoprotective	EGCG	Vero cells (ATCC CCL-81)	Alteration of cellular redox milieu	(47)
Neuroprotective	EGCG	transgenic G93A ALS model mice	Decrease of death signals, GSK3β, cyto c, caspase-3	(48)

Table 3. Molecular targets and inhibition of the proinflammatory biomarkers based on in vitro research and molecular simulation studies.

Drug targets	Compound	Mechanism	Reference
3CLpro/Mpro/Nsp5	EGCG, ECG, GCG TF2, TF3	Mpro-polyphenol complex (in silico docking) BE (EGCG):-7.6kcal/mol TF2: -9.8 kcal/mol TF3:-10 kcal/mol	(49)
PL^{pro}	EGCG	In silico docking studies BE:-8.601 kcal/mol	(50)
RdRp	EGCG, TF2a, TF2b, TF3	In silico studies	(51)
S protein RBD (PDB: 6VXX)	EGCG, TF	In silico studies	(52)
ACE2	EGCG, TF3	Binding with ACE2 receptor prevents viral entry, regulating ACE 2 expression	(53)
IL6	EGCG	MIF inhibition causing anti-inflammatory effect	(54)
IL-1β	EGCG	blocks IL-1β	(55)
IL-2	EGCG	Inhibits IL-2 proprietary α chains promoting T-cell regulation	(56)
IFN-γ	EGCG	Binds to SEB, neutralizes it and inhibits IFN-γ and IL-2	(57)

Tea polyphenols as anti-viral agents

Research has indicated that tea polyphenols possess antiviral capabilities against multiple viruses, such as COVID-19(34–37). The body's immunity against COVID-19 and other viral infectious diseases can be strengthened by tea polyphenol (34). Tea polyphenols have the ability to inhibit COVID-19 through a number of mechanisms, such as activating transcription factors, blocking cellular receptors, and inhibiting multiple viral targets(34). Polyphenols have an antiviral effect because of their ability to disrupt host cell defence by controlling the mitogen-activated protein kinase signal, or because of their interaction with viral protein and/or RNA through the benzene ring. Tea polyphenols have the ability to decrease vascular permeability by blocking neutrophil migration across the endothelial cell monolayer(34). Two tea-derived polyphenols, theaflavins from black tea and epigallocatechin-3-gallate (EGCG) from green tea, have been reviewed for their antiviral properties (35). It has been found that EGCG inhibits hepatitis C virus entry(38). It has been observed that theaflavin-3,3'-digallate (TF3) inhibits the virus's hemagglutinin by preventing the virus from adhering to MDCK cells (39). Tea polyphenols can also prevent and intervene in COVID-19 through the gut-lung axis(36, 37). Several such studies stating the antiviral capabilities of polyphenols have been given in Table 1 with their mechanism of action.

Multifaceted strengths with the intake of dietary polyphenols

Epidemiological studies and related meta-analyses suggest that long-term intake of diets high in polyphenols may offer protection against the development of certain chronic diseases, such as diabetes, osteoporosis, neurological diseases, and cardiovascular diseases (53-54). Tables 2 and 3 describe the in

vitro, in vivo, and in silico studies conducted to determine the effectiveness of various polyphenols against various disease targets and their mechanisms of action. The beneficial effects of polyphenols seem to be mediated through their interactions with related machinery and cellular signaling pathways that regulate cell function in both healthy and diseased states. The capacity of dietary polyphenols to lessen the effects of oxidation on the human body, shield organs and cell structure from degradation, and preserve their functional integrity are just a few of their advantages (55).

Neurodegenerative diseases

Polyphenols have been reported to have neuroprotective properties and can contribute to reduced outcomes in neuropsychiatric disorders (56). In addition to enhancing hippocampus neurogenesis, learning abilities, memory, and mood, they can also enhance language, verbal memory, visual, and cognitive functions (56, 57). In older adults, polyphenols have the ability to improve verbal fluency, psychomotor abilities, attention speed, episodic memory, and overall cognitive performance while also modulating cerebral hemodynamics and resting regional cerebral blood flow. Pure herbal compounds, including green tea polyphenol (-)-epigallocatechin-3-gallate, resveratrol, curcumin, quercetin, and others, as well as extracts rich in polyphenols that exhibit the most promising neuroprotective effects, have been reviewed in terms of their therapeutic potential (58).

Antioxidant properties

Polyphenols act as antioxidants in the body, that is, they aid in defending the body and scavenging free radicals that harm cells (59). By scavenging reactive oxygen species (ROS) and modifying signaling pathways, they can improve cardiovascular system health outcomes and shield people from cardiovascular diseases. Polyphenols have the capacity to inhibit neuroinflammation, shield neurons from damage brought on by neurotoxins, and enhance memory, learning, and cognitive function, among other neuroprotective effects (60).

Cancer prevention

Polyphenols have been shown to have potential preventive and therapeutic effects for cancer(61). They can modulate multiple molecular events involved in carcinogenesis and have anti-inflammatory properties. Some of the polyphenols that have been studied for their anticancer properties including green tea polyphenol (–) epigallocatechin-3-O-gallate (EGCG), curcumin, resveratrol, apigenin, quercetin, and kaempferol (62). By scavenging ROS, polyphenols have the ability to prevent the growth of tumors, cause apoptosis in cancerous cells, and impede the evolution of malignancies. To increase the chemo-preventive effects of traditional chemotherapy, this type of therapeutic approach can be applied. In order to better cancer treatment and control with natural products, polyphenols can regulate important components of cancer signaling pathways. This emphasizes the significance of having a thorough grasp of these controls (63).

Cardiovascular health

It is commonly believed that polyphenols can prevent cardiovascular disease (64). Diets high in polyphenols may be linked to a lower prevalence of cardiovascular diseases, including myocardial infarction and coronary heart disease, according to epidemiological research. These substances' antithrombotic, anti-inflammatory, and anti-aggregative qualities can enhance endothelial function and prevent platelet aggregation. BPolyphenols like resveratrol, curcumin, and epigallocatechin gallate (EGCG) have been demonstrated to improve cardiovascular health by lowering reactive oxygen species (ROS) and changing signaling pathways (65). P A diet rich in polyphenols reduces the risk of cardiovascular disease, its complications, and the associated mortality. Polyphenol-rich cuisines include the French diet, which emphasizes red wine consumption, the diets of the Far East, which stress the drinking of green tea, the South Asian diet, which

largely uses turmeric, and the Mediterranean diet, which emphasizes olive oil use (66).

Safety and toxicity of tea polyphenols

Despite the fact that pre-clinical model systems have demonstrated the cancer-prevention potential of tea and its polyphenols. But it's crucial to consider these substances' toxicity and safety, especially when taking large doses or using them over an extended length of time. Several clinical studies have investigated the safety and pharmacokinetics of tea polyphenols in humans. In one such study involving 40 individuals, researchers administered 800mg EGCG once/day, 400mg EGCG twice/day, 800 mg EGCG as Polyphenol E once/day, 400mg EGCG as Polyphenol E twice/day or a placebo to the 5 groups (8 individuals per group). According to the study's findings, a healthy person can safely take tea polyphenol products for four weeks at doses equal to the EGCG found in eight to sixteen cups of green tea, either once a day or in divided doses twice a day. TThe researchers also found that chronic green tea polyphenol treatment at a high daily bolus dose (800 mg EGCG or Polyphenon E once daily) increases the systemic availability of free EGCG by more than 60% (67).

In a 2018 scientific opinion, the EFSA ANS Panel examined 12 clinical studies in which participants received EGCG. The panel concluded that, as long as EGCG intake is kept to a daily maximum of 300 mg, catechins from traditional green tea infusions and reconstituted drinks with a composition equivalent to traditional infusions are generally regarded as safe under the presumption of safety. A statistically significant increase in serum transaminase, a marker of liver injury, has been observed in response to doses of EGCG taken as a food supplement of 800 mg or more per day, according to the panel's analysis of the 12 clinical studies. (68).

In a preliminary study, adult patients with solid tumors were given oral green tea extract at doses ranging from 0.5 to 5.05g/m^2. The majority of these dose levels demonstrated mild to moderate toxicities, which were quickly reversed when the green tea extract was stopped. The highest amount that could be tolerated was 4.2 g/m^2 per day, or 1 g/m^2 three times a day. Caffeine-related toxicities, such as gastrointestinal and neurological effects, were the dose-limiting toxicities (69). Whereas EGCG levels did not accumulate or seem to be dose related, pharmacokinetic analyses revealed that caffeine levels accumulated in a dose-dependent manner. Overall, the available evidence suggests that green tea polyphenols are generally safe and well-tolerated when administered at recommended doses. However, caution should be exercised when administering high doses of these compounds, particularly in individuals with pre-existing liver or gastrointestinal conditions. Further research is needed to fully understand the safety and toxicity of green tea polyphenols in humans (70).

An alternative approach in the preparation of sanitizers

Currently, there are countless measures that are being taken in multiple ways to prevent the entry of viruses. One of the strategies majorly includes the use of alcohol-based sanitizers which in turn prove to be useful in disinfecting the skin against the micro-organisms. They are marketed under several brand labels and made available at every corner (71). However, when it comes to the formulation of these products there are still certain issues that need to be addressed with respect to their overall effectiveness, toxicity of certain ingredients, environmental pollution, and the short-term duration of their anti-microbial activity with their use(72). Therefore, there is an urgent need for an alternative approach that could certainly aid in the effectiveness, increase its longevity and at same time remain nontoxic and pro environmental. Considering the issue, are the group of compounds tea polyphenols, particularly, epigallocatechin-3-gallate (EGCG) and its lipophilic derivatives. Based on literature studies, they are found to be effective against a wide range of

viral infections and have been proven to contain novel inhibitory and anti-viral properties (73). Hence, they appear to be strong contenders in developing alternative methods for formulating sanitizers with improved and better version thereby, providing long term anti-microbial effects.

Summary and future perspectives

The SARS-Co-V-2 and the identified variants remain to be the deadliest form of the viral agents, reported to have caused millions of deaths worldwide. The uncontrolled transmission of the virus via respiratory droplets and aerosols requires in depth research regarding the host-viral interactions. As a solution to combat the deadly virus, several synthetic drugs have been approved by the FDA on an urgent basis. Moreover, persistent administration of these drugs mediates immunosuppression. Also, these being synthetic in nature have been reported to have multiple side effects restricting their use during the course of the disease. In many cases, patients have been known to report other complications that include nausea, dizziness, increased uric acid levels in blood, decreased white blood cell count particularly, neutrophils, cardiac diseases, abnormal liver function tests, etc (74). Thus, the intake of higher dosage of these drugs makes the patient to have a higher probability to acquire other chronic diseases. This eventually, leads to falling into a loop where the individual becomes prone to long term illness. Regardless of the caveats, these approved drugs remain to be the only class of medicines as of now, amidst the alarming spread of the viral diseases. Research work is still in progress to comprehend about the behavior and spread of the virus along with their variants which would help in formulating broad spectrum anti-viral drugs (75). Furthermore, future studies are needed to address the amalgamative application of natural and synthetic compounds providing synergistic effects. In continuation with our purpose, the following areas necessitate to delve into the employment of polyphenols as accessory compounds against the viral infections.

i) To determine whether the molecular simulation studies can be duplicated onto animal models and further translated to human subjects. ii) To identify the possible link between tea consumption (black or green) and decreased rate of acquiring the viral infections mainly the novel coronavirus. iii) To evaluate the effectiveness of tea polyphenols as added dietary supplement and immunity boosting agents that aid against fighting the viral infections by performing thorough in vivo and cohort studies, since an effective immune system is the only structured way to overcome these diseases. iv) To implement deeper understanding in the development of combinatorial drugs comprising of natural and synthetic origins with a higher dosage of plant-based compounds and their derivatives (76).

Conclusion

The Covid-19 outbreak has turned to an international health emergency with several efforts being made worldwide to contain the spread of the disease. In this study, we shortly outline the collective health benefits with the intake of tea along with an emphasis on the prophylaxis and treatment of viral infections adding tea polyphenols as potential adjuvants in dealing against viral infections. Although, this does not in any way certainly promote their solo usage. They could only be used as supplementary agents and nutraceuticals along with the available set of precautionary measures and medications. Nonetheless, polyphenols and their derivatives in combination with the other anti-viral drugs could possibly aid in designing a novel set of medicines having both natural and synthetic origin. This would facilitate the switch to a better and improved class of drugs with lesser toxicity and better efficacy.

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Conflict of Interest

The authors declare no conflict of interest.

References

1. www.who.int. (2020). *Clinical management of severe acute respiratory infection when novel coronavirus (nCoV) infection is suspected*.

2. McIntosh, K. and Perlman, S. (2015). Coronaviruses, Including Severe Acute Respiratory Syndrome (SARS) and Middle East Respiratory Syndrome (MERS). *Mandell, Douglas, and Bennett's Principles and Practice of Infectious Diseases*, 1928-1936.

3. Zheng, J. (2020). SARS-CoV-2: an Emerging Coronavirus that Causes a Global Threat. *International Journal of Biological Sciences*, 16(10), 1678–1685.

4. Chen, N., Zhou, M., Dong, X., Qu, J., Gong, F., Han, Y., Qiu, Y., Wang, J., Liu, Y., Wei, Y., Xia, J., Yu, T., Zhang, X. and Zhang, L. (2020). Epidemiological and clinical characteristics of 99 cases of 2019 novel coronavirus pneumonia in Wuhan, China: a descriptive study. *The Lancet*, 395(10223), 507–513.

5. Bn, S., S, S. and Rk, S. (2011). Green Tea Catechin, epigallocatechin-3-gallate (EGCG): Mechanisms, Perspectives and Clinical Applications. *Biochemical Pharmacology.* 82(12), 1807-21.

6. Xu, J., Xu, Z. and Zheng, W. (2017). A Review of the Antiviral Role of Green Tea Catechins. *Molecules*, 22(8), 1337.

7. Cascella, M., Rajnik, M., Cuomo, A., Dulebohn, S.C. and Di Napoli, R. (2023). *Features, Evaluation and Treatment Coronavirus (COVID-19)*. https://www.ncbi.nlm.nih.gov/books/NBK554776/.

8. Letko, M., Marzi, A. and Munster, V. (2020). Functional assessment of cell entry and receptor usage for SARS-CoV-2 and other lineage B betacoronaviruses. *Nature Microbiology*, 5(4), 562–569.

9. Parasher, A. (2020). COVID-19: Current understanding of its pathophysiology, clinical presentation and treatment. *Postgraduate Medical Journal*, 97(1147), 312–320.

10. Wu, A., Peng, Y., Huang, B., Ding, X., Wang, X., Niu, P., Meng, J., Zhu, Z., Zhang, Z., Wang, J., Sheng, J., Quan, L., Xia, Z., Tan, W., Cheng, G. and Jiang, T. (2020). Genome Composition and Divergence of the Novel Coronavirus (2019-nCoV) Originating in China. *Cell Host & Microbe*, 27(3), 325–328.

11. Gao, Y., Yan, L., Huang, Y., Liu, F., Zhao, Y., Cao, L., Wang, T., Sun, Q., Ming, Z., Zhang, L., Ge, J., Zheng, L., Zhang, Y., Wang, H., Zhu, Y., Zhu, C., Hu, T., Hua, T., Zhang, B. and Yang, X. (2020). Structure of the RNA-dependent RNA polymerase from COVID-19 virus. *Science*, 368(6492).

12. Hancock, A.S., Stairiker, C.J., Boesteanu, A.C., et al., (2018). Transcriptome Analysis of Infected and Bystander Type 2 Alveolar Epithelial Cells during Influenza A Virus Infection Reveals In Vivo Wnt Pathway Downregulation. *Journal of Virology*, 92(21).

13. Qian, Z., Travanty, E.A., Oko, L., Edeen, K., Berglund, A., Wang, J., Ito, Y., Holmes, K.V. and Mason, R.J. (2013). Innate Immune Response of Human Alveolar Type II Cells Infected with Severe Acute Respiratory Syndrome–Coronavirus. *American Journal of Respiratory Cell and Molecular*

Biology, 48(6), 742–748.

14. Kubina, R. and Dziedzic, A. (2020). Molecular and Serological Tests for COVID-19. A Comparative Review of SARS-CoV-2 Coronavirus Laboratory and Point-of-Care Diagnostics. *Diagnostics*, 10(6), 434.

15. Lippi, G. and Plebani, M. (2020). The critical role of laboratory medicine during coronavirus disease 2019 (COVID-19) and other viral outbreaks. *Clinical Chemistry and Laboratory Medicine.* 58(7), 1063-1069.

16. Chousterman, B.G., Swirski, F.K. and Weber, G.F. (2017). Cytokine storm and sepsis disease pathogenesis. *Seminars in Immunopathology*, 39(5), 517–528.

17. Liu, J., Li, S., Liu, J., et al., (2020). Longitudinal characteristics of lymphocyte responses and cytokine profiles in the peripheral blood of SARS-CoV-2 infected patients. *EBioMedicine*, 55, 102763.

18. Wang, Q. and Lu, F. (2020). Correlation analysis between disease severity and inflammation-related parameters in patients with COVID-19: a retrospective study. *BMC Infectious Diseases*, 20(1).

19. Velavan, T.P. and Meyer, C.G. (2020). Mild versus severe COVID-19: laboratory markers. *International Journal of Infectious Diseases.* 95, 304-307.

20. Xu, Z., Shi, L., Wang, Y., et al., (2020). Pathological findings of COVID-19 associated with acute respiratory distress syndrome. *The Lancet Respiratory Medicine*, 45(8).

21. Ghosh, R., Chakraborty, A., Biswas, A. and Chowdhuri, S. (2020). Evaluation of green tea polyphenols as novel corona virus (SARS CoV-2) main protease (Mpro) inhibitors – an in silico docking and molecular dynamics simulation study. *Journal of Biomolecular Structure and Dynamics*, 1–13.

22. Chinnappan, S., Wen, K.J., Sally, L., Kuang, F.W., Yin, Y.B. and Mani, R.R. (2023). Nutrition Importance in preventing COVID-19 pandemic disease. *Current Trends in Biotechnology and Pharmacy*, 17(4A (Supplement)), 98–107.

23. Lin, D., Zheng, Y., Huang, Y., et al., (2020). Investigation of the structural, physical properties, antioxidant, and antimicrobial activity of chitosan- nano-silicon aerogel composite edible films incorporated with okara powder. *Carbohydrate Polymer*, 250, 116842–116842.

24. Gupta, S.C., Tyagi, A.K., Deshmukh-Taskar, P., Hinojosa, M., Prasad, S. and Aggarwal, B.B. (2014). Downregulation of tumor necrosis factor and other proinflammatory biomarkers by polyphenols. *Archives of Biochemistry and Biophysics*, 559, 91–99.

25. Lim, H., Min, D.S., Park, H. and Kim, H.P. (2018). Flavonoids interfere with NLRP3 inflammasome activation. *Toxicology and Applied Pharmacology*, 355, 93–102.

26. Hong, M., Cheng, L., Liu, Y., Wu, Z., Zhang, P. and Zhang, X. (2022). A Natural Plant Source-Tea Polyphenols, a Potential Drug for Improving Immunity and Combating Virus. *Nutrients*, 14(3), 550.

27. Mhatre, S., Srivastava, T., Naik, S. and Patravale, V. (2020). Antiviral activity of green tea and black tea polyphenols in prophylaxis and treatment of COVID-19: A review. *Phytomedicine*, 153286.

28. Xiang, Q., Cheng, L., Zhang, R., Liu, Y., Wu, Z. and Zhang, X. (2022). Tea Polyphenols Prevent and Intervene in COVID-19 through Intestinal Microbiota. *Foods*, 11(4), 506.

29. Xu, L., Ho, C.-T., Liu, Y., Wu, Z. and Zhang, X. (2022). Potential Application of Tea Polyphenols to the Prevention of COVID-19 Infection: Based on the Gut-Lung Axis. *Frontiers in Nutrition*, 9.

30. Wang, X., Dong, W., Zhang, X., Zhu, Z., Chen, Y., Liu, X. and Guo, C. (2021). Antiviral Mechanism of Tea Polyphenols against Porcine Reproductive and Respiratory Syndrome Virus. *Pathogens*, 10(2), 202–202.

31. Jae Min Song (2018). Anti-infective potential of catechins and their derivatives against viral hepatitis. *Clinical and Experimental Vaccine Research*, 7(1), 37–37.

32. Bhardwaj, V.K., Singh, R., Sharma, J., Rajendran, V., Purohit, R. and Kumar, S. (2020). Identification of bioactive molecules from tea plant as SARS-CoV-2 main protease inhibitors. *Journal of Biomolecular Structure and Dynamics*, 1–10.

33. Vázquez-Calvo, Á., Jiménez de Oya, N., Martín-Acebes, M.A., Garcia-Moruno, E. and Saiz, J.-C. (2017). Antiviral Properties of the Natural Polyphenols Delphinidin and Epigallocatechin Gallate against the Flaviviruses West Nile Virus, Zika Virus, and Dengue Virus. *Frontiers in Microbiology*, 8.

34. Yang, Z.-F., Bai, L.-P., Huang, W., Li, X.-Z., Zhao, S.-S., Zhong, N.-S. and Jiang, Z.-H. (2014). Comparison of in vitro antiviral activity of tea polyphenols against influenza A and B viruses and structure–activity relationship analysis. *Fitoterapia*, 93, 47–53.

35. Raekiansyah, M., Buerano, C.C., Luz, M.A.D. and Morita, K. (2018). Inhibitory effect of the green tea molecule EGCG against dengue virus infection. *Archives of Virology*, 163(6), 1649–1655.

36. Weber, C., Sliva, K., von Rhein, C., Kümmerer, B.M. and Schnierle, B.S. (2015). The green tea catechin, epigallocatechin gallate inhibits chikungunya virus infection. *Antiviral Research*, 113, 1–3.

37. Patrick Reid, St., Shurtleff, A.C., Costantino, J.A., Tritsch, S.R., Retterer, C., Spurgers, K.B. and Bavari, S. (2014). HSPA5 is an essential host factor for Ebola virus infection. *Antiviral Research*, 109, 171–174.

38. Chowdhury, P., Sahuc, M.-E., Rouillé, Y., et al., (2018). Theaflavins, polyphenols of black tea, inhibit entry of hepatitis C virus in cell culture. *PLOS One*, 13(11), e0198226.

39. Cory, H., Passarelli, S., Szeto, J., Tamez, M. and Mattei, J. (2018). The Role of Polyphenols in Human Health and Food Systems: A Mini-Review. *Frontiers in Nutrition*, 5(87).

40. Rudrapal, M., Khairnar, S.J., Khan, J., et al., (2022). Dietary Polyphenols and Their Role in Oxidative Stress-Induced Human Diseases: Insights into Protective Effects, Antioxidant Potentials and Mechanism(s) of Action. *Frontiers in Pharmacology*, 13.

41. Rathod, N.B., Elabed, N., Punia, S., Ozogul, F., Kim, S.-K. and Rocha, J.M. (2023). Recent Developments in Polyphenol Applications on Human Health: A Review with Current Knowledge. *Plants*, 12(6), 1217.

42. Izabela Grabska-Kobyłecka, Szpakowski, P., Aleksandra Król, Dominika Książek-Winiarek, Andrzej Kobyłecki, Andrzej Głąbiński and Nowak, D. (2023). Polyphenols and Their Impact on the Prevention of Neurodegenerative Diseases and Development. *Nutrients*, 15(15), 3454–3454.

43. García-Aguilar, A., Palomino, O., Benito, M. and Guillén, C. (2021). Dietary Polyphenols in Metabolic and Neurodegenerative Diseases: Molecular Targets in Autophagy and Biological Effects. *Antioxidants*,

10(2), 142.

44. Ammar, A., Trabelsi, K., Boukhris, O., Bouaziz, B., Müller, P., M Glenn, J., Bott, N.T., Müller, N., Chtourou, H., Driss, T. and Hökelmann, A. (2020). Effects of Polyphenol-Rich Interventions on Cognition and Brain Health in Healthy Young and Middle-Aged Adults: Systematic Review and Meta-Analysis. *Journal of Clinical Medicine*, 9(5), 1598.

45. Spagnuolo, C., Napolitano, M., Tedesco, I., Moccia, S., Milito, A. and Russo, G.L. (2016). Neuroprotective Role of Natural Polyphenols. *Current Topics in Medicinal Chemistry*, 16(17), 1943–1950.

46. Zhang, H. and Tsao, R. (2016). Dietary polyphenols, oxidative stress and antioxidant and anti-inflammatory effects. *Current Opinion in Food Science*, 8, 33–42.

47. Di Lorenzo, C., Colombo, F., Biella, S., Stockley, C. and Restani, P. (2021). Polyphenols and Human Health: The Role of Bioavailability. *Nutrients*, 13(1), 273.

48. Bešlo, D., Golubić, N., Rastija, V., Agić, D., Karnaš, M., Šubarić, D. and Lučić, B. (2023). Antioxidant Activity, Metabolism, and Bioavailability of Polyphenols in the Diet of Animals. *Antioxidants*, 12(6), 1141.

49. Kim, K.H., Ki, M.-R., Min, K.H. and Pack, S.P. (2023). Advanced Delivery System of Polyphenols for Effective Cancer Prevention and Therapy. *Antioxidants*, 12(5), 1048.

50. Cháirez-Ramírez, M.H., de la Cruz-López, K.G. and García-Carrancá, A. (2021). Polyphenols as Antitumor Agents Targeting Key Players in Cancer-Driving Signaling Pathways. *Frontiers in Pharmacology*, 12.

51. Ding, S., Xu, S., Fang, J. and Jiang, H. (2020). The Protective Effect of Polyphenols for Colorectal Cancer. *Frontiers in Immunology*, 11.

52. Sharma, A., Kaur, M., Katnoria, J.K. and Nagpal, A.K. (2018). Polyphenols in Food: Cancer Prevention and Apoptosis Induction. *Current Medicinal Chemistry*, 25(36), 4740–4757.

53. Goszcz, K., Duthie, G.G., Stewart, D., Leslie, S.J. and Megson, I.L. (2017). Bioactive polyphenols and cardiovascular disease: chemical antagonists, pharmacological agents or xenobiotics that drive an adaptive response? *British Journal of Pharmacology*, 174(11), 1209–1225.

54. Alotaibi, B.S., Ijaz, M., Buabeid, M., Kharaba, Z.J., Yaseen, H.S. and Murtaza, G. (2021). Therapeutic Effects and Safe Uses of Plant-Derived Polyphenolic Compounds in Cardiovascular Diseases: A Review. *Drug Design, Development & Therapy*, 15, 4713–4732.

55. Khurana, S., Venkataraman, K., Hollingsworth, A., Piche, M. and Tai, T.C. (2013). Polyphenols: Benefits to the Cardiovascular System in Health and in Aging. *Nutrients*, 5(10), 3779–3827.

56. Habauzit, V. and Morand, C. (2011). Evidence for a protective effect of polyphenols-containing foods on cardiovascular health: an update for clinicians. *Therapeutic Advances in Chronic Disease*, 3(2), 87–106.

57. Zhang, L., Gui, S., Wang, J., Chen, Q., Zeng, J., Liu, A., Chen, Z. and Lu, X. (2020). Oral administration of green tea polyphenols (TP) improves ileal injury and intestinal flora disorder in mice with Salmonella typhimurium infection via resisting inflammation, enhancing antioxidant action and preserving tight junction. *Journal of Functional Foods*, 64, 103654.

58. Murugesu, S., Balan, T., Tamri, N.I.M., Zamree, L.N.H., Samba, N.F.Z., Anuar, S.N.H.M., Fatinathan, S. and Perumal, V. (2024). Molecular Docking Analysis of Christanoate and Christene from Christia vespertilionis Plants as Potential Inhibitors of Covid-19. *Current Trends in Biotechnology and Pharmacy*, 18(2), 1736–1744.

59. Si, H., Fu, Z., Pon, Wei, Z., et al., (2011). Dietary Epicatechin Promotes Survival of Obese Diabetic Mice and Drosophila melanogaster. *Journal of Nutrition*, 141(6), 1095–1100.

60. Takemoto, M., Takemoto, H. and Saijo, R. (2016). Theaflavin Synthesized in a Selective, Domino-Type, One-Pot Enzymatic Biotransformation Method with *Camellia sinensis* Cell Culture Inhibits Weight Gain and Fat Accumulation to High-Fat Diet-Induced Obese Mice. *Biological & Pharmaceutical Bulletin*, 39(8), 1347–1352.

61. Ahmed, N.A., Radwan, N.M., Aboul Ezz, H.S. and Salama, N.A. (2016). The antioxidant effect of Green Tea Mega EGCG against electromagnetic radiation-induced oxidative stress in the hippocampus and striatum of rats. *Electromagnetic Biology and Medicine*, 36(1), 1–11.

62. Khalatbary, A.R. and Ahmadvand, H. (2011). Anti-Inflammatory Effect of the Epigallocatechin Gallate Following Spinal Cord Trauma in Rat. *Iranian Biomedical Journal*, 15(1-2), 31–37.

63. Zhou, Y., Tang, J., Du, Y., Ding, J. and Liu, J.-Y. (2016). The green tea polyphenol EGCG potentiates the antiproliferative activity of sunitinib in human cancer cells. *Tumor Biology*, 37(7), 8555–8566.

64. Huang, C.-Y., Han, Z., Li, X., Xie, H.-H. and Zhu, S.-S. (2017). Mechanism of EGCG promoting apoptosis of MCF-7 cell line in human breast cancer. *Oncology Letters*, 14(3), 3623–3627.

65. Pase, M.P., Scholey, A.B., Pipingas, A., Kras, M., Nolidin, K., Gibbs, A., Wesnes, K. and Stough, C. (2013). Cocoa polyphenols enhance positive mood states but not cognitive performance: a randomized, placebo-controlled trial. *Journal of Psychopharmacology*, 27(5), 451–458.

66. Stringer, T.P., Guerrieri, D., Vivar, C. and van Praag, H. (2015). Plant-derived flavanol (−)epicatechin mitigates anxiety in association with elevated hippocampal monoamine and BDNF levels, but does not influence pattern separation in mice. *Translational Psychiatry*, 5(1), e493.

67. Pola, M., Tiwari, A. and Chandrasai, P.D. (2023). A Comprehensive Review on Technological Advances in Alternate Drug Discovery Process: Drug Repurposing. *Current Trends in Biotechnology and Pharmacy*, 17(2), 907–916.

68. Wang, W., Zhang, Z.-Z., Wu, Y., et al., (2018). (–)-Epigallocatechin-3-Gallate Ameliorates Atherosclerosis and Modulates Hepatic Lipid Metabolic Gene Expression in Apolipoprotein E Knockout Mice: Involvement of TTC39B. *Frontiers in Pharmacology*, 9(9).

69. Chourasia, M., Koppula, P.R., Battu, A., Ouseph, M.M. and Singh, A.K. (2021). EGCG, a Green Tea Catechin, as a Potential Therapeutic Agent for Symptomatic and Asymptomatic SARS-CoV-2 Infection. *Molecules*, 26(5), 1200.

70. Singh, S., Sk, M.F., Sonawane, A., Kar, P. and Sadhukhan, S. (2020). Plant-derived natural polyphenols as potential antiviral drugs against SARS-CoV-2 via RNA-dependent RNA polymerase (RdRp) inhibition: an in-silico analysis. *Journal of Biomolecular Structure and Dynamics*, 39(16), 1–16.

71. Tallei, T.E., Tumilaar, S.G., Niode, N.J., Fatimawali, Kepel, B.J., Idroes, R., Effendi, Y., Sakib, S.A. and Emran, T.B. (2020). Potential of Plant Bioactive Compounds as SARS-CoV-2 Main Protease (Mpro) and Spike (S) Glycoprotein Inhibitors: A Molecular Docking Study. *Scientifica*, 2020, 1–18.

72. Maiti, S. and Banerjee, A. (2020). Epigallocatechin gallate and theaflavin gallate interaction in SARS-CoV-2 spike-protein central channel with reference to the hydroxychloroquine interaction: Bioinformatics and molecular docking study. *Drug Development Research*, 82(1), 86–96.

73. Akhtar, N. and Haqqi, T.M. (2011). Epigallocatechin-3-gallate suppresses the global interleukin-1beta-induced inflammatory response in human chondrocytes. *Arthritis Research & Therapy*, 13(3), R93.

74. Wang, J., Pae, M., Meydani, S.N. and Wu, D. (2012). Epigallocatechin-3-Gallate Inhibits Expression of Receptors for T Cell Regulatory Cytokines and Their Downstream Signaling in Mouse CD4+ T Cells. *The Journal of Nutrition*, 142(3), 566–571.

75. Younes, M., Aggett, P., Aguilar, F., et al., (2018). Scientific opinion on the safety of green tea catechins. *EFSA Journal*, 16(4).

76. Hsu, S. (2015). Compounds Derived from Epigallocatechin-3-Gallate (EGCG) as a Novel Approach to the Prevention of Viral Infections. *Inflammation & Allergy-Drug Targets*, 14(1), 13–18.

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Identification of Phytoconstituents and investigation of nephroprotective potential of seeds of *Rumex vesicarius* in experimental animals

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Abstract

The present study is planned for phytochemical analysis and to evaluate the nephroprotective effect of methanol seed extract of *Rumex vesicarius* on cisplatin-induced nephrotoxicity. The study initiated with pharmacognostic studies of seeds followed by preparation of methanol extract by using hot extraction method. Then the preliminary phytochemical studies were performed followed by HPLC and GC-MS analysis. Further *in vitro* antioxidant studies were performed by adopting DPPH, ABTS and NO techniques. Further Pharmacological studies were initiated with acute toxicity studies. Nephroprotective activity in cisplatin induced model is evaluated by the determination of levels of serum markers and urinary functional parameters. Phytochemical analysis revealed the presence of bioactive phytoconstituents. HPLC revealed in the presence of rutin, whereas GC-MS analysis of methanolic extract resulted in 108 compounds. Upon *in vitro* antioxidant studies the seed extract exhibited markable antioxidant activity. Animal studies showed that methanol extract successfully ameliorated the nephrotoxicity induced by cisplatin in dose dependent passion. Hereby the current study resulted in identification of phytochemical compounds in seeds of *Rumex vesicarius* and also scientifically validated the ethnomedicinal use of seeds of *Rumex vesicarius* in attenuation of renal problems.

Keywords: Nephrotoxicity, Cisplatin, *Rumex vesicarius,* Phytochemical.

Introduction

Even with the rapid advancement of molecular medicine, the search for natural remedies and the discovery of novel phytochemicals remain vast areas of study. Since antiquity, family or cultural tradition has dictated how to consume therapeutic plants. This practise has been transmitted through the centuries and is now common in folk medicine. (1,2). Numerous studies on herbal medicines are being undertaken constantly because they are widely accepted by people as a healthy alternative to treatments based on chemicals or genetics (3). There are numerous functional secondary metabolites found in medicinal plants throughout the plant, but many of these compounds' biological activities are still unknown (4).

In traditional medicine wide number of medicinal plants are used to treat many diseases and disorders including renal problems and many of them are not evaluated scientifically. *Rumex vesicarius* L., is one such plant. It is a

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member of the Polygonaceae family and has green, edible leaves. The abundance of ß-carotenes, vitamins, proteins, lipids, organic acids, and minerals in this shrub makes it a valuable nutritional supplement (5). The plant is used to treat tumours, hepatic disorders, bad digestion, constipation, heart problems, aches, illnesses of the spleen, bloating, hiccough, asthma, bronchitis, dyspepsia, dermatitis, toothache, and nausea (6). The entire plant is medicinally noteworthy and heals a number of maladies.

Prominently seeds of this plant is used to cure urinary and renal troubles by tribal people of various regions of India (7). Hence the current study was aimed at the identification of phytoconstituents and evaluation of the nephroprotective activity of seeds of *Rumex vesicarius* in cisplatin- induced rat model.

Materials and Methods

Plant material collection and authentication

Plant material (Seeds of *Rumex vesicarius*) was obtained from a local market in Tirupati, Andhra Pradesh, then authenticated by botanist Dr. K. Madhava Chetty, an assistant professor in the department of botany at S.V. University, Tirupati. A voucher specimen (No. 0911) was subsequently submitted.

Pharmacognostic studies

Powder microscopy was carried out as per the standard methodology by treating with different chemical reagents and identification of diagnostic characters (8). Seed powder was subjected for determination of varied physicochemical parameters as per the procedures mentioned in Ayurvedic Pharmacopoeia 1996 (9).

Extract preparation

The seeds were ground up into a coarse powder, and the extract was made by macerating them with methanol and then extracting them using a hot extraction process (10).

Preliminary phytochemical screening

Preliminary phytochemical studies was performed for the methanol extract of seeds of *Rumex vesicarius* (MERV) to screen the presence of various phytoconstituents as per standard procedures (11).

Quantitative estimation of Phenols and Flavonoids

Tannic acid was used as a reference to evaluate the extracts' total phenolic content using the Folin-Ciocalteu reagent (12). Utilizing aluminium chloride colorimetry, it was possible to quantify the total flavonoid concentration. Using established techniques, the extract's flavonoid content (quercetin equivalent) was determined (13).

HPLC analysis

HPLC of the crude extract was performed on column C18 Phenomenox 5u,4.6×250 mm. The mobile phase used for the analysis was methanol and water; injection volume: 20 **µ**l; flow rate: 1ml/min and detected at 254nm.

GC-MS analysis

The "Clarus 680 GC. Gas chromatograph fitted and linked to a mass detector Turbo mass gold-Perkin Elmer with turbomass version 5.2.0 spectrometer with an Elite-5MS (5% Phenyl 95% dimethyl Polysilioxane), 30m x 500m id capillary column" was used to conduct the GC-MS analysis of the MERV. The instrument's initial temperature setting was 600C. The oven's temperature was then increased to 300^0C. at the rate of hike of 10^0C/min, and maintained for 6min. Helium flow rate: 1.0ml/min; Ionization voltage: 70eV. Injection of samples in split mode at 1:10. The component spectrums were compared to the database of component spectrums in the GC-MS NIST library. Further bioactivity of the compounds was predicted based on Dr. Dukes ethnomedicinal database (14).

In vitro antioxidant studies

DPPH antioxidant activity

(2, 2-Diphenyl-1-picrylhydrazyl (DPPH) assay

MERV in concentrations from 1 to 5 mg/mL was taken and method adopted for DPPH antioxidant activity was as per Olamide et al., 2017 (15). Ascorbic acid (Vitamin C) was used standard.

ABTS (2,2'-azino-bis (3-ethylbenzothiazoline-6-sulphonic acid) radical scavenging activity

The extract's ability to scavenge ABTS was compared to that of ascorbic acid, and % inhibition was calculated using the accepted techniques (16).

Nitric oxide scavenging (NOS) assay

The NOS assay was conducted by using reagents like sodium nitroprusside, Griess reagent etc by methods of Reddy et al., 2020 (17).

Pharmacological screening of nephroprotective activity

Animals

The current study made use of healthy Wistar strain albino rats that were 2 to 3 months old and weighed 150 to 200g. The experiment was conducted in accordance with the Institutional Animal Ethical Committee's (IAEC) approval and Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA) of India's requirements (Registration No. Ref No. 1677/PO/Res/S/2012/CPCSEA/IAEC/14/23-02-19).

Acute toxicity studies

Acute toxicity studies were performed as per OECD 423 guidelines (18).

Treatment protocol [19]:

Five groups of six animals each were formed out of the animals. Animals in Group I were given the vehicle orally from days 1 through 9 and were retained as a standard control. Animals in the Group-II were kept as disease controls and received a single dose of cisplatin (6 mg/kg/b.w., i.p.) on day 5 and vehicle (water) orally from days 1 through 9. Animals in Group III were given methanol extract (200 mg/kg b.w.) from Day 1 through Day 9, as well as cisplatin (6 mg/kg b.w., i.p.) on Day 5 and were retained as Lower dose treated animals. Animals in Group IV were given methanol extract (400 mg/kg b.w.) from days 1 through 9, as well as cisplatin (6 mg/kg b.w., i.p.) on days 5 and were kept as higher dose treated animals. Animals in Group V were given Cystone (5 ml/kg b.w.) from Days 1 through 9 and Cisplatin (6 mg/kg b.w., i.p.) on Day 5 and were treated as Standard.

At the end of treatment, assessment of nephrotoxicity is done by estimation of urinary functional parameters, serum biochemical tests and Anti-oxidant studies to the isolated kidney tissue as per standard methodology (10). Statistical analysis carried out and the mean $\pm$ standard error was used to express the data. Tukey-Kramer multiple comparison tests and one-way ANOVA were conducted, and mean values with $p<0.05$ were regarded as significant.

Results and Discussion

Pharmacognostic studies

The morphological characters were examined and seeds found to be light brown in colour, odourless, slight sweet in taste and conical in shape. Powder microscopy of seed powder showed palisade cells, pitted xylem vessels, starch grains, annular xylem vessels, pitted tracheids and parenchyma cells with cotyledons (Figure 1). Different physicochemical parameters, such as ash values, extractive values, and loss on drying, were calculated and tabulated in Table 1.

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Figure 1: Powder microscopy of seed powder of *Rumex vesicarius*

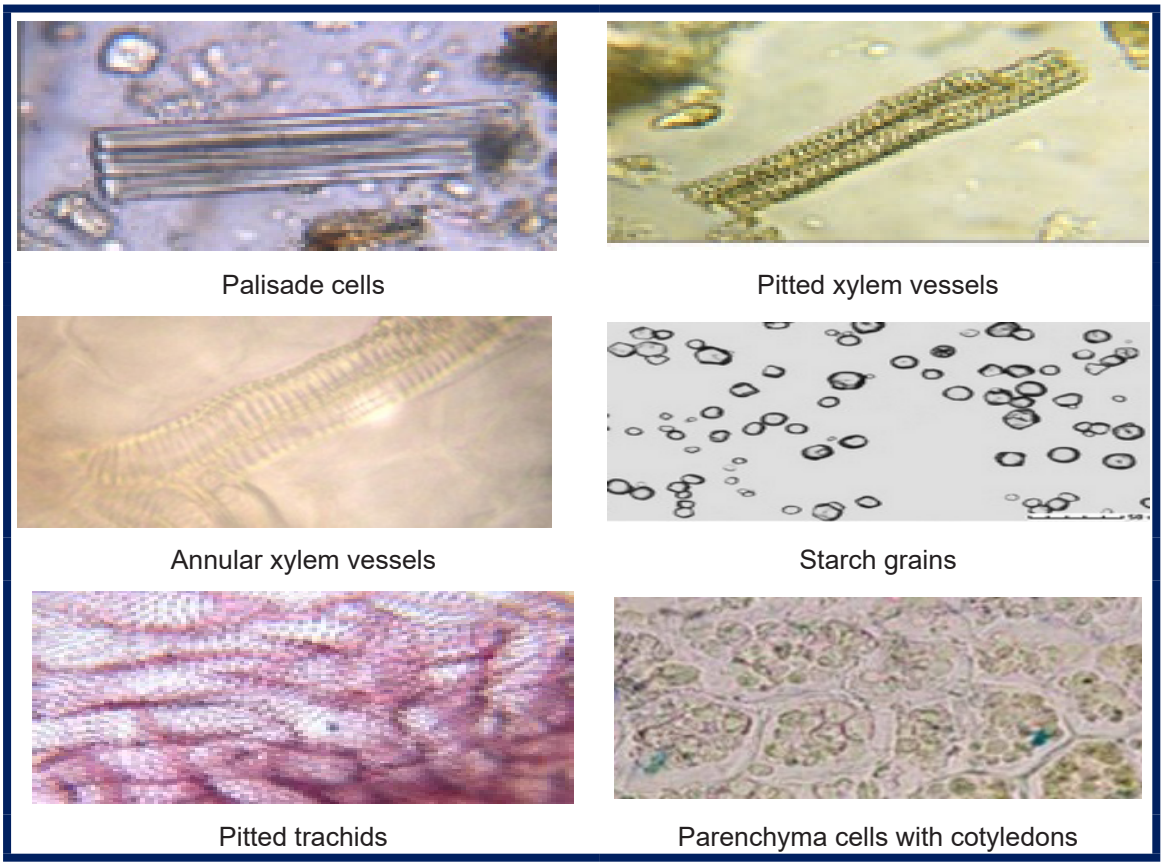

Table 1: Physicochemical analysis of seeds of *Rumex vesicarius*

S. No	Parameters	Values (%w/w)
1.	Ash values a)Total ash value b)Acid insoluble ash c)Water insoluble ash	1.4 0.504 1.14
2.	Extractive values a)Alcohol soluble extractive b)Water soluble extractive c)chloroform soluble extractive	 **72** **42** **20**
3.	Loss on drying	**4.2**

Screening for preliminary phytochemicals and quantitative estimate

Preliminary phytochemical screening of MERV revealed the presence of phenolic substances such as saponins, triterpinoids, glycosides, and flavonoids. The Phenolic content was calculated as mg of gallic acid /g of plant material and it was found to be 64.80±1.50. The total flavonoid content of MERV were demonstrated as quercetin equivalents per gram and was discovered to be 50.08±0.12 mg of quercetin equivalent /g.

HPLC analysis of of MERV

HPLC analysis of MERV resulted in 20 number of peaks at different retention times ranging from 3.13 to 23.86 (Figure 2). Out of all

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the peaks, peak eluted at 5.16 (retention time) in MERV was matched with rutin (Std) **(**Figure:2).

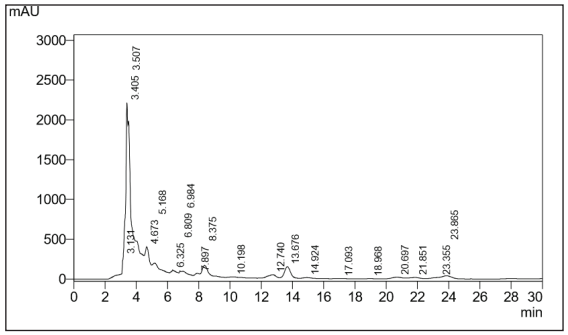
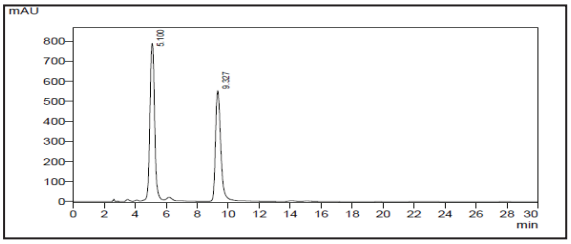


Figure 2a: HPLC chromatogram of MERVFig-



ure 2b: HPLC chromatogram of RUTIN

In Vitro antioxidant studies

Antioxidant studies of MERV was determined by three different assays and results were tabulated in Table 4. IC_{50} values of MERV is 3.33 (DPPH), 2.57 (NOS) and 3.58 (ABTS). The scavenging activity was increased with the escalation in the concentrations of MERV (Table 2).

GC-MS analysis of MERV

The GC-MS analysis of MERV resulted in 108 compounds (Figure 3). Among them many of the compounds like 2,3-Anhydro-d-mannosan, N-butyl-N-vinylacetamide, 2-hydroxy-gamma-butyrolactone, P-Dioxane-2,3-diol, 4-Tosyl-diformal-1-rhamnitol are biologically active and may strongly provide innate defense against oxidative stress.

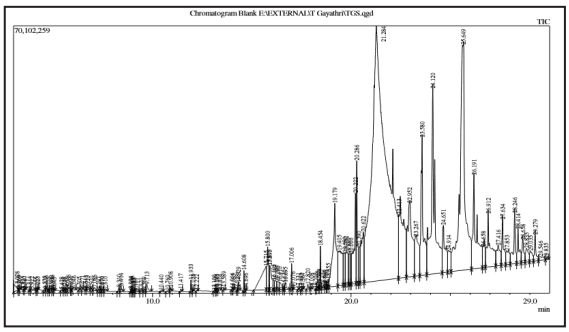


Figure 3: GC-MS chromatogram of MERV

Table :2 Effect of MERV on *in vitro* antioxidants

Concentration (mg/ml)	% inhibition of DPPH (Mean±SEM)	% inhibition NOS (Mean±SEM)	%inhibition ABTS (Mean±SEM)
1	41.84±0.30	42.63±0.48	36.56±0.48
2	42.28±0.52	50.93±0.59	41.16±0.56
3	52.90±0.67	52.4±0.66	52.43±0.69
4	52.92±0.77	53.2±0.69	53.2±0.74
5	54.22±0.69	54.26±0.75	54±0.86
Standard (Vitamin C)	43.19±0.14	55.2±0.12	55.66±0.15
IC_{50}	3.33	2.57	3.58

Table 4: Effect of MERV on oxidative stress

Group	**Catalase (**uM/min/mg protein)	**SOD** **(**Units/mg protein)	**GSH** (µmol/mg protein)	**MDA (**nmol/mg protein)
I	45.58±0.52	26.75±0.66	12.42±0.48	43.58±1.46
II	17.38±0.37[a*]	8.25±0.30 [a*]	3.50±0.26 [a*]	98.75±0.54 [a*]
III	31.45±0.51[b*]	11.95±0.19 [b*]	4.1±0.33 [b*]	79.14±0.13 [b*]
IV	38.91±0.38[b*]	15.13±0.31 [b*]	6.9±0.20 [b*]	69.53±0.58 [b*]
V	43 .67±0.40 [a* b*]	17.25±0.61 [a* b*]	8.66±0.24 [a* b*]	55.82±0.13 [a* b*]

All values were expressed as mean±SEM of 6 observations
a*=P<0.05, considered statistically significant when compared to the normal sgroup.
b*=P<0.05, considered statistically significant when compared to the disease control.

Pharmacological evaluation

Acute toxicity studies

There was no morbidity and animals had not shown any changes in behavior and no signs of toxicity. Hence, the MERV was found to be safe at dose of 2000 mg/kg, b.w.

Effect of MERV on cisplatin-induced nephrotoxicity

When compared to normal animals, the administration of cisplatin led to a significantly higher (p 0.05) level of BUN, serum creatinine, urinary total protein, lipid peroxidation, and a lower level of urinary creatinine, as well as lower levels of reduced glutathione, catalase, and superoxide dismutase. However, treatment of the MERV considerably counteracted the effects of cisplatin in dose-dependent passion at both doses of 200 and 400 mg/kg,b.w (Table 5 and 6). Furthermore, the illness control group also showed a considerable reduction in body weight. When compared to disease control, the extract-treated animals displayed a significant reduction in the decline of body weight.

Table 3: Effect of MERV on biochemical parameters of Cisplatin –induced nephrotoxicity

Group	Serum creatinine(mg/dl)	Blood urea nitrogen(mg/dl)	Urine creatinine(mg/dl)	Total urinary protein(g/dl)
I	0.84±0.01	11.67±0.44	8.89±0.28	4.16±0.24
II	2.57±0.06 [a*]	28±0.38 [a*]	2.49±0.26 [a*]	11.93±0.26 [a*]
III	1.73±0.05[b*]	18.35±0.29 [b*]	5.65±0.27 [b*]	8.89±0.21 [b*]
IV	1.27±0.05 [b*]	12.50±0.24 [b*]	10.67±0.21 [b*]	5.56±0.27 [b*]
V	1.18±0.04[a*b*]	15.42±0.35 [a*b*]	9.33±0.44 [a*b*]	5.192±0.21 [a*b*]

All values were expressed as mean±SEM of 6 observations
a*=P<0.05, considered statistically significant when compared to the normal group.
b*=P<0.05, considered statistically significant when compared to the disease control.

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Table 5: Effect of MERV on body weight in cisplatin- induced nephrotoxicity

Group	Body Weight	
	Initial	Final
I	163.8±0.60	169.85±0.60
II	155±0.96[a*]	133.8±0.61 [a*]
III	145±0.98 [b*]	139±0.96 [b*]
IV	161±0.86 [b*]	152±0.89 [b*]
V	159.2±0.60 [a* b*]	151.8±0.62 [a* b*]

All values were expressed as mean±SEM of 6 observations
a*=P<0.05, considered statistically significant when compared to the normal group.
b*=P<0.05, considered statistically significant when compared to the disease control.

Discussion

Rumex vesicarius is one of several anti-oxidant-rich medicinal herbs. Tribes from several parts of India utilize its seeds as folk medicine to cure urological and renal issues. The goal of the current study was to assess the nephroprotective potential of *Rumex vesicarius* seeds. To evaluate the quality and purity of plant material, the work began with powder microscopy and was followed by physico-chemical analysis of *Rumex vesicarius* seeds. The presence of flavonoids and phenolic chemicals, which are known to have effective antioxidant and nephroprotective properties, was discovered in methanol extract by preliminary phytochemical analyses (20).

The phenolic and flavonoid content of MERV was found to be high overall. Rutin was detected in the MERV extract after additional HPLC analysis. According to earlier scientific research, rutin protects the kidneys against drug-induced nephrotoxicity (21).

A variety of bioactive chemicals were found in the *Rumex vesicarius* seed extract after GC-MS analysis and were discovered to have powerful antioxidant characteristics. The existence of phytochemical antioxidants was also confirmed by other in vitro antioxidant tests. According to earlier findings, plants with a high concentration of antioxidant phytochemicals are beneficial in reducing nephrotoxicity.

Nephrotoxicity is a serious consequence of the effective anticancer medication cisplatin and a dose-limiting side effect (22). An increase in serum indicators, urine total protein, and malondialdehyde levels, as well as a decrease in urinary creatinine, GSH, SOD, and catalase levels, were all signs of renal impairment brought on by the administration of cisplatin. According to studies by Fang et al. (2021), Yadav et al. (2019), these results are consistent (23,24).

As described in earlier investigations on different plants, treatment with MERV reduced the effects of cisplatin on dose-dependent passion. This might be brought on by MERV's "free radical scavenging activity, enhanced glomerular filtration, and rejuvenation in renal tubular cells." Innate protection against oxidative stress brought on by cisplatin may be significantly attributed to the presence of antioxidant principles such flavonoids, particularly rutin, and other phytoconstituents.

Conclusion

The current scientific research resulted in identification of various phytochemical compounds in seeds of *Rumex vesicarius.* Furthermore, the current study presents scientific evidence that supports the ethnomedicinal use of *Rumex vesicarius* seeds in the amelioration of renal issues.

References

1. Mohammadhosseini M., Frezza C., Venditti A., Mahdavi B.(2021) An overview of the genus Aloysia Paláu (Verbenaceae): Essential oil composition, ethnobotany and biological activities. *Nat. Prod. Res.*1–17. [PubMed] [Google Scholar]

2. Omer Iqbal M, Bashir Yahya E, Andleeb S, Masood Ahmed M, Umar Javaid M, Sha-

keel W, Iqbal I.(2021) In vivo assessment of reversing Cisplatin-Induced nephrotoxicity using *Jatropha mollissima* crude extract and its potential cytotoxicity. Saudi J Biol Sci. 28(12):7373-7378. doi: 10.1016/j.sjbs.2021.08.057. PMID: 34867040; PMCID: PMC8626311.

3. Barbosa H.M., Albino A.M., Cavalcante F.S.A., Lima R.A.(2017) Abordagem fitoquímica de metabólitos secundários em Solanum acanthodes (Solanaceae) Hook. *South Am. J. Basic Education, Technical Technological*;4(1) [Google Scholar]

4. Abogmaza, A. F., Keer, K. F., Ayad, A. T., & Yahya, E. B. A Review on the Medicinal and Aromatic Plants Growing in Libya and Their Therapeutic Properties. Int. Res. J. Sci. Technol., 2(1).

5. Beddou F, Bekhechi C, Ksouri R, Chabane Sari D, Atik Bekkara F.(2015) Potential assessment of Rumex vesicarius L. as a source of natural antioxidants and bioactive compounds. J Food Sci Technol;52(6):3549-60. doi: 10.1007/s13197-014-1420-9. PMID: 26028737; PMCID: PMC4444908.

6. Gopal R, Vijayakumaran M, Venkatesan R, Kathiroli S (2008) Marine organisms in Indian medicine and their future prospects. Nat Prod Radiance 7(2):139–145. http://nopr.niscair.res.in/bitstream/123456789/5658/1/NPR%207(2)%20139-145.pdf

7. Mostafa HAM, ElBakry AA, Alam EA. (2011) Evaluation of antibacterial and antioxidant activities of different plant parts of *Rumex vesicarius* L. (Polygonaceae) *Int J Pharm Pharm Sci*;3(2):109–118. [Google Scholar]

8. Prakash, A., Janmeda, P., Pathak, P. *et al.* (2019) Development and standardization of quality control parameters of different parts of *Trianthema portulacastrum* L.. *SN Appl. Sci.* **1:**1108 . https://doi.org/10.1007/s42452-019-1074-3

9. The Ayurvedic Pharmacopoeia of India, Part- I, Volume – III, Government of India, Ministry of Health and Family Welfare. Department of Ayush; New Delhi; 123.

10. Adikay S, Pravallika BV and Sruth KS (2021) Phytochemical analysis and pharmacological evaluation of ethanol extract of fruits of Grewia tiliaefolia for nephroprotective activity. Int J Pharm Sci & Res 12(9): 4933-41. doi: 10.13040/IJPSR.0975-8232.12(9):4933-41.

11. Harborne J B,(1973) Phytochemical Methods, Chapman & Hall: International Edition, Japan: Toppan Company Ltd, 49-188.

12. Sumana Dutta, Sanjib Ray(2020) Comparative assessment of total phenolic content and in vitro antioxidant activities of bark and leaf methanolic extracts of Manilkara hexandra (Roxb.) Dubard, Journal of King Saud University - Science,32: 643-647, ISSN 1018-3647, https://doi.org/10.1016/j.jksus.2018.09.015.

13. Aryal S, Baniya MK, Danekhu K, Kunwar P, Gurung R, Koirala N(2019) Total Phenolic Content, Flavonoid Content and Antioxidant Potential of Wild Vegetables from Western Nepal. Plants (Basel);8(4):96. doi: 10.3390/plants8040096. PMID: 30978964; PMCID: PMC6524357.

14. Matulyte I, Marksa M, Ivanauskas L, Kalvėnienė Z, Lazauskas R, Bernatoniene J(2019) GC-MS Analysis of the Composition of the Extracts and Essential Oil from *Myristica fragrans* Seeds Using Magnesium Aluminometasilicate as Excipient. Molecules ;24(6):1062. doi: 10.3390/molecules24061062. PMID: 30889896; PMCID: PMC6471610.

15. Olamide E. Adebiyi, Funsho O. Olayemi,

Tan Ning-Hua, Zeng Guang-Zhi(2017) In vitro antioxidant activity, total phenolic and flavonoid contents of ethanol extract of stem and leaf of Grewia carpinifolia, Beni-Suef University Journal of Basic and Applied Sciences, 6(1):10-14,ISSN:2314-8535,https://doi.org/10.1016/j.bjbas.2016.12.003. (https://www.sciencedirect.com/science/article/pii/S2314853516301007)

16. Shahinuzzaman, M., Yaakob, Z., Anuar, F.H. *et al*(2020) In vitro antioxidant activity of *Ficus carica* L. latex from 18 different cultivars. *Sci Rep* **10,** 10852 . https://doi.org/10.1038/s41598-020-67765-1

17. Reddy KS, Likithasree P, Peraman R, Jyothi MV, Babu CN, Pradeepkumar B, Sudheer A(2020) Spatial long-term memory retention by banana and papaya peel extract: In silico and in vivo evaluation. International Journal of Pharmaceutical Investigation;10(2):202-7.

18. Organization for Economic Cooperation and Development (OECD). Guideline 423 for testing chemicals: Paris 2001: 1-14.

19. Zahra Eslamifar, Abbas Moridnia, Susan Sabbagh, Reza Ghaffaripour, Leila Jafaripour, Mahin Behzadifard(2021) "Ameliorative Effects of Gallic Acid on Cisplatin-Induced Nephrotoxicity in Rat Variations of Biochemistry, Histopathology, and Gene Expression", BioMedResearch International, Article ID 2195238, 11 https://doi.org/10.1155/2021/2195238

20. Hossain MA, AL-Raqmi KA, AL-Mijizy ZH, Weli AM, Al-Riyami Q(2013) Study of total phenol, flavonoids contents and phytochemical screening of various leaves crude extracts of locally grown Thymus vulgaris. Asian Pac J Trop Biomed;3(9):705-10. doi: 10.1016/S2221-1691(13)60142-2. PMID: 23998010; PMCID: PMC3757278.

21. Qu S, Dai C, Lang F, Hu L, Tang Q, Wang H, Zhang Y, Hao Z(2018) Rutin Attenuates Vancomycin-Induced Nephrotoxicity by Ameliorating Oxidative Stress, Apoptosis, and Inflammation in Rats. Antimicrob Agents Chemother;63(1):e01545-18. doi: 10.1128/AAC.01545-18. PMID: 30397060; PMCID: PMC6325182.

22. McSweeney KR, Gadanec LK, Qaradakhi T, Ali BA, Zulli A, Apostolopoulos V(2021) Mechanisms of Cisplatin-Induced Acute Kidney Injury: Pathological Mechanisms, Pharmacological Interventions, and Genetic Mitigations. Cancers (Basel);13(7):1572. doi: 10.3390/cancers13071572. PMID: 33805488; PMCID: PMC8036620.

23. Fang, Cy., Lou, Dy., Zhou, Lq. *et al*(2021) Natural products: potential treatments for cisplatin-induced nephrotoxicity. *Acta Pharmacol Sin* **42:**1951–1969 https://doi.org/10.1038/s41401-021-00620-9

24. Yadav AV and Upasani CD(2019) Nephroprotective activity of Plumeria rubra against cisplatin induced nephrotoxicity in experimental rats. International Journal of Pharmacy and Pharmaceutical Sciences; 11: 108-13.

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GC-MS Analysis and Quantification of Some Secondary Metabolites of the Algerian *Phragmites australis* Leaf Extract and Their Biological Activities

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Abstract

This study aims to assess the biological impacts and phytochemical compounds present in the leaves of *Phragmites australis*. Standard protocols were used for qualitative and quantitative chemical screening, where DPPH and FRAP assays were used to estimate the antioxidant activity. Regarding the anti-inflammatory potential, it was tested through the red blood cells' protection against hemolysis and the protection of protein from denaturation. Using GC-MS, volatile compounds can be identified. Results show that more than 200 volatile compounds in this plant were identified by the GC-MS analysis including 1-Dodecanol (48.25%), Pyrazine, tetramethyl (1.28%), Heptadecane, 2,6,10,15-tetramethyl (0.91%), Cyclopentasiloxane, decamethyl (0.93%) and Cyclotetrasiloxane, octamethyl (0.56%). Qualitative phytochemical tests show the richness of the aqueous extract of different biocompounds such as polyphenols, flavonoids, terpenoids, tannins, and alkaloids. Total phenolic content and total flavonoids exhibit paramount concentration (70,74±1,94 mg GA eq/ g dry extract and 3,64±0,98 mg Q eq/ g dry extract). Results reveal a moderate antioxidant activity compared to the anti-inflammatory one whose values are prominent. We conclude that *P. australis* is a promising source of bioactive compounds that could be exploited in the pharmaceutical field.

Keywords: *P. australis;* GC-MS; Polyphenols; Antioxidant; Anti-inflammatory; IC_{50}.

Introduction

Plants continue to be a major source of new physiologically active chemicals. Many medicinal plant species are being evaluated for biological activity due to the growing interest in phytomedicine (1). The phytochemical components of medicinal plants are responsible for the wide spectrum of pharmacological effects (2). *Phragmites australis*, or common reed, is a wetland species known for its abundance of bioactive components in aqueous plant extracts such as tannins, phenolic compounds, flavonoids, terpenoids, and glycosides (3). The genus Phragmites contains more than ten species in the world (4). It is used in traditional medicine to cure a variety of human and livestock ailments (5). Aquatic extracts of *P. australis* rhizomes were shown to have antioxidant and hepatopro-

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tective effects. Furthermore, leaf extract has anti-melanogenesis and antioxidant properties (6, 7). Another study reported the antiviral activity of the plant aqueous extract (8). The intention of this study is to identify the phytochemical profile of *Phragmites australis'* leaves using GC-MS analysis and to figure out the medicinal effectiveness of the Algerian *P. australis* regarding inflammation and oxidative stress disorders.

Materials and methods

Plant material and extraction method

Phragmites australis specimens (figure 1) were gathered in October 2022 from Touggourt state in Algeria. Leaves were separated from specimens and cleaned with water to eliminate dust and debris and left to dry out in the shade before being ground to fine powder. Leaves' aqueous extract was prepared as follows: 5 g of *Phragmites australis* leaves' powder was added to 50 ml of distilled water. The solution was exposed to a temperature of 50° C while stirring it for two hours. The preparation was then left to be macerated for 24 hours at room temperature. After filtring with a muslin cloth and filter paper, the filtrate was dried in a laboratory oven and saved in the freezer for further use (9).

Figure 1: leaves of *Phragmites australis*

Phytochemical analyses

P. australis leaf extract was analyzed for the presence of several compounds such as phenols, tannins, alkaloids, steroids, saponins, flavonoids, and terpenoids using conventional protocols for phytochemical screening (10-12).

Determination of total phenol content

The total phenolic contents (TPC) of *P. australis* leaf extract was determined by the Folin Ciocalteu method (13).100 µl of the extract is mixed with 500 µl of the FC reagent and 400 µl of Na_2CO_3 at 7.5% (w / v). After 10 minutes of being stirred and incubated in the dark at room temperature, the absorbance of the solution is measured at 760 nm using a UV spectrophotometer. The results are expressed in mg gallic acid equivalent/ g of dry extract with reference to the calibration curve of gallic acid.

Determination of total flavonoid content

The total flavonoid contents (TFC) of *P. australis* leaf extract was determined as described by (Dehpour A et al., 2009) (14). 500 µl of the extract, 100 µl $AlCl_3$, 100µl of 1 M sodium acetate, and 2.8 ml of distilled water. The mixture is incubated in the dark and at room temperature for 30 minutes after being well stirred. The blank is made by replacing the extract with 95% methanol and the absorbance is measured at 415nm using a UV spectrophotometer. The results are expressed in mg equivalent quercetin / g of dry extract material with reference to the quercetin calibration curve.

Gas chromatography and mass spectroscopy

Plant extract of leaves was prepared in universal solvent methanol for which 1µl plant extract was employed to quantify the volatile compounds by GCMS analysis. For extraction of volatiles headspace solid-phase micro-extraction (SPME) with DVB/CAR/PDMS fiber was used. Firstly, the fiber was conditioned in the GC injection port at 270°C for 4 h. Then the fiber was put into the vial with the sample using

an adapter for 15 min at room temperature. After that, the fiber was put into the injection port of a gas chromatograph for desorption. Desorption time was 10 min at 260°C in the splitless mode. For analysis was used a 7890A GC system (Agilent Technologies, Santa Clara, United States) coupled with a 5975C VL Triple-Axis mass detector (Agilent Technologies, Santa Clara, United States). Separation was run on a DB-5MS capillary column (25 m × 0.2 mm; 0.33 µm film thickness; J&W, Folsom, California) with helium as a carrier gas at a flow rate of 0.6 mL/min. The temperature of the injector and transfer line were 260°C and 280°C, respectively. The oven program of temperature was: the initial temperature at 40°C was held for 3 min, then increased at 4°C/min to 160°C and further increased at 10°C/min to 280°C, with the final temperature held for 3 min. The masses were scanned from 33 to 333 Da. The ionization energy value was set to 70 eV. The result interpretation of GCMS data was evaluated using the National Institute of Standards and Technology (NIST) database. The comparative assessment assisted in identifying unknown chemicals when compared to the stored NIST library in order to investigate the available plant extract data. The molecular chemical data was derived by determining the name, formula, weight, and structure of the sample's volatile components.

Data presentation and analysis

The results were expressed as mean ± standard deviation (SD), calculated from duplicate determinations and the linear relationship was visually determined.

In vitro biological activities

The anti-oxidant activity is measured using two assays: DPPH free-radical scavenging activity as described by Nwidu et *al.* (15) and the ferric reducing anti-oxidant power assay (FRAP) as described by Oyaizu (16); while the anti-inflammatory effect of *Phragmites australis* and biosynthesized copper NPs was investigated *in vitro* using two methods. The first one is a measure of protein denaturation inhibition as described by Vennila, et *al*. (17), and the second one is a measure of red blood cells protection against hemolysis as stated by Vinjamuri et al. (18).

Results and Discussion

Phytochemical screening

The phytochemical analyses show that leaf extract is rich in different active components (Table 1). A (+) indicates the presence of phytochemicals, whereas a (-) indicates its absence.

Table 1**:** Phytochemical screening of *P. australis* leaves aqueous extract.

Phytochemical compound	***P. australis* leaves aqueous extract**
Polyphenols	+
Flavoniods	+
Alkaloids	+
Tanins	+
Saponins	+
Terpenoids	+
Reducing compounds	+

Total phenolic content is expressed in terms of gallic acid equivalents (mg GA eq/ g of dry plant extract), whereas total flavoniods content is expressed in terms of quercetin equivalents (mg Q eq/ g of dry plant extract), using the following equation based on the calibration curve: Y= 0,007x+0,009 (R2=0,985) for phenolic compound and Y=0,006x+0,031 for flavonoids compounds (R2=0,993). The results are represented in Table 2.

Table 2: Total phenol and flavonoid contents in *P. australis* leaves aqueous extract.

Compounds	**Total phenol content mg GA eq/ g dry extract**	**Total flavonoid content mg Q eq/ g dry extract**
P. australis leaves aqueous extract	70.74±1.94	3.64±0.98

GC-MS analysis

The screening and the identification of volatile compounds of *P. australis* leaf extract were conducted through GC-MS chromatogram (Fig. 2). Using the GC-MS technique, more than 200 components were identified (Table 3). The chemical constituents of *P. australis* were dominated by 1-Dodecanol (48.25%), Pyrazine, tetramethyl (1.28%), Heptadecane, 2,6,10,15-tetramethyl (0.91%), Cyclopentasiloxane, decamethyl (0.93%) and Cyclotetrasiloxane, octamethyl (0.56%).

Table 3: Quantification of volatile compounds by GC-MS of *P. australis* leaf extract.

Peak #	Name	Formula	R.T. (s)	Area %
	Pyridine, 2,4,6-trimethyl-	$C_8H_{11}N$	519.375	0.185
	Cyclotetrasiloxane, octamethyl-	$C_8H_{24}O_4Si_4$	525.98	0.566
	1-Hexanol, 2-ethyl-	$C_8H_{18}O$	560.425	0.198
	Cyclohexene, 4-ethenyl-1,4-dimethyl-	$C_{10}H_{16}$	563.796	0.179
	Pyrazine, tetramethyl-	$C_8H_{12}N_2$	623.786	1.287
	Linalyl acetate	$C_{12}H_{20}O_2$	637.587	0.071
	Nonanal	$C_9H_{18}O$	641.938	0.088
	Cyclopentasiloxane, decamethyl-	$C_{10}H_{30}O_5Si_5$	691.491	0.939
	Undecane	$C_{11}H_{24}$	738.646	0.049
	Cyclodecanol	$C_{10}H_{20}O$	744.805	0.054
	1,5-Dimethyl-1-vinyl-4-hexenyl butyrate	$C_{14}H_{24}O_2$	792.808	0.057
	m-Ethylacetophenone	$C_{10}H_{12}O$	804.602	0.007
	Dodecane, 2,6,11-trimethyl-	$C_{15}H_{32}$	816.363	0.029
	(1R,3R,4S,5S)-1-Isopropyl-4-methylbicyclo[3.1.0]hexan-3-yl acetate-rel	$C_{12}H_{20}O_2$	829.467	0.031
	Dodecane, 2,6,11-trimethyl-	$C_{15}H_{32}$	833.846	0.081
	Undecanal	$C_{11}H_{22}O$	841.331	0.019
	Trimethylsilylcatecholpyruvatetris(trimethylsilyl) ether	$C_{21}H_{40}O_5Si_4$	856.708	0.49
	Triacetin	$C_9H_{14}O_6$	875.414	0.201
	Tridecane, 5-methyl-	$C_{14}H_{30}$	882.295	0.02
	Pentanoic acid, 2,2,4-trimethyl-3-hydroxy-, isobutyl ester	$C_{12}H_{24}O_3$	887.91	0.305
	Dodecane, 3-methyl-	$C_{13}H_{28}$	890.952	0.092
	Pentanoic acid, 2,2,4-trimethyl-3-hydroxy-, isobutyl ester	$C_{12}H_{24}O_3$	905.368	0.477
	Octadecane, 6-methyl-	$C_{19}H_{40}$	922.984	0.015
	Dodecanal	$C_{12}H_{24}O$	931.787	0.063
	Bicyclo[5.2.0]nonane, 2-methylene-4,8,8-trimethyl-4-vinyl-	$C_{15}H_{24}$	953.746	0.04
	1-Dodecanol	$C_{12}H_{26}O$	986.66	48.251
	2,5-Cyclohexadiene-1,4-dione, 2,6-bis(1,1-dimethylethyl)-	$C_{14}H_{20}O_2$	988.172	0.083
	Ethanone, 1-(6,6-dimethylbicyclo[3.1.0]hex-2-en-2-yl)-	$C_{10}H_{14}O$	999.155	0.031

	3-Ethoxy-1,1,1,7,7,7-hexamethyl-3,5,5-tris(trimethylsiloxy) tetrasiloxane	$C_{17}H_{50}O_7Si_7$	1005.56	0.363
	Tetracontane, 3,5,24-trimethyl-	$C_{43}H_{88}$	1007.09	0.186
	Oxirane, dodecyl-	$C_{14}H_{28}O$	1017.19	0.026
	Dodecanoic acid, methyl ester	$C_{13}H_{26}O_2$	1026.9	0.123
	Ethyl dodecyl ether	$C_{14}H_{30}O$	1032.92	0.086
	Lilial	$C_{14}H_{20}O$	1036.4	0.039
	Hexadecane	$C_{16}H_{34}$	1041.66	0.028
	Decane, 5-propyl-	$C_{13}H_{28}$	1044.73	0.015
	Benzene, (1-propylheptyl)-	$C_{16}H_{26}$	1048.45	0.016
	Dodecane, 5-methyl-	$C_{13}H_{28}$	1049.38	0.019
	Hexane, 3,3-dimethyl-	C_8H_{18}	1060.39	0.011
	Heptadecane, 2,6,10,14-tetramethyl-	$C_{21}H_{44}$	1064.15	0.03
	2-Methyl-1-undecanol	$C_{12}H_{26}O$	1080.98	0.19
	1-Iodo-2-methylundecane	$C_{12}H_{25}I$	1086.69	0.185
	Diethyl Phthalate	$C_{12}H_{14}O_4$	1087.61	0.164
	Diphenyl sulfide	$C_{12}H_{10}S$	1088.24	0.025
	trans-2-Dodecen-1-ol	$C_{12}H_{24}O$	1097.83	0.01
	Benzene, (1-butylheptyl)-	$C_{17}H_{28}$	1117.73	0.036
	Cyclopentaneacetic acid, 3-oxo-2-pentyl-, methyl ester	$C_{13}H_{22}O_3$	1132.68	0.243
	Octane, 1,1'-oxybis-	$C_{16}H_{34}O$	1135.74	0.056
	3-Isopropoxy-1,1,1,7,7,7-hexamethyl-3,5,5-tris(trimethylsiloxy) tetrasiloxane	$C_{18}H_{52}O_7Si_7$	1138.43	0.355
	Tetradecane, 2,2-dimethyl-	$C_{16}H_{34}$	1140.89	0.018
	Benzene, (1-ethylnonyl)-	$C_{17}H_{28}$	1141.76	0.021
	n-Hexyl salicylate	$C_{13}H_{18}O_3$	1152.93	0.025
	1-Iodo-2-methylundecane	$C_{12}H_{25}I$	1162.12	0.159
	Heptadecane, 2,6,10,14-tetramethyl-	$C_{21}H_{44}$	1166.71	0.11
	Hydrazinecarboxamide	CH_5N_3O	1186.1	0.012
	Undecane, 4,6-dimethyl-	$C_{13}H_{28}$	1194.08	0.051
	n-Hexadecanoic acid	$C_{16}H_{32}O_2$	1203.45	0.041
	Hydrazinecarboxamide	CH_5N_3O	1225.78	0.006
	1-Iodo-2-methylundecane	$C_{12}H_{25}I$	1233.77	0.109
	Hexadecane, 2,6,10,14-tetramethyl-	$C_{20}H_{42}$	1240.95	0.079
	Benzene, (1-methylundecyl)-	$C_{18}H_{30}$	1243.14	0.017
	Benzoic acid, 2-hydroxy-, 2-methylbutyl ester	$C_{12}H_{16}O_3$	1245.87	0.013
	13-Methyltetradecanal	$C_{15}H_{30}O$	1246.8	0.026
	Isoamyllaurate	$C_{17}H_{34}O_2$	1265.1	0.411

	Dodecanoic acid, 1,1-dimethylpropyl ester	$C_{17}H_{34}O_2$	1267.63	0.081
	Caffeine	$C_8H_{10}N_4O_2$	1275.72	0.017
	Hydrazinecarboxamide	CH_5N_3O	1281.58	0.002
	(1R,2R,4S)-2-(6-Chloropyridin-3-yl)-7-methyl-7-azabicyclo[2.2.1]heptane	$C_{12}H_{15}ClN_2$	1282.25	0.008
	Pentadecane	$C_{15}H_{32}$	1302.02	0.05
	Benzenemethanol, α-[1-(ethylmethylamino)ethyl]-, [R-(R*,S*)]-	$C_{12}H_{19}NO$	1312.82	0.105
	Benzene, (1-methylnonadecyl)-	$C_{26}H_{46}$	1313.44	0.017
	Hydrazinecarboxamide	CH_5N_3O	1326	0.024
	n-Hexadecanoic acid	$C_{16}H_{32}O_2$	1340.11	0.075
	Dibutyl phthalate	$C_{16}H_{22}O_4$	1348.07	0.146
	Cyclooctasiloxane, hexadecamethyl-	$C_{16}H_{48}O_8Si_8$	1355.79	0.173
	Dodecane, 4,6-dimethyl-	$C_{14}H_{30}$	1367.08	0.022
	Semicarbazide	CH_5N_3O	1395.08	0.006
	Hydrazinecarboxamide	CH_5N_3O	1419.5	0.001
	Nickel tetracarbonyl	C_4NiO_4	1440.13	0.001
	Hydrazinecarboxamide	CH_5N_3O	1443.12	0.008
	3-Ethoxy-1,1,1,7,7,7-hexamethyl-3,5,5-tris(trimethylsiloxy) tetrasiloxane	$C_{17}H_{50}O_7Si_7$	1449.59	0.121
	dl-Alanyl-l-alanine	$C_6H_{12}N_2O_3$	1451.96	0.003
	Semicarbazide	CH_5N_3O	1458.17	0.001
	Hydrazinecarboxamide	CH_5N_3O	1466.48	0.001
	Benzenemethanol, α-(1-aminoethyl)-	$C_9H_{13}NO$	1468.36	0.002
	Semicarbazide	CH_5N_3O	1512.72	0.002
	Hexasiloxane, tetradecamethyl-	$C_{14}H_{42}O_5Si_6$	1534.62	0.108
	Hydrazinecarboxamide	CH_5N_3O	1548.59	0.007
	dl-Alanyl-l-alanine	$C_6H_{12}N_2O_3$	1558.04	0.001
	Hydrazinecarboxamide	CH_5N_3O	1564.48	0.009
	dl-Alanyl-l-alanine	$C_6H_{12}N_2O_3$	1566.33	0.001
	Hydrazinecarboxamide	CH_5N_3O	1581.87	0.006
	Semicarbazide	CH_5N_3O	1602.56	0.002
	Benzoic acid, octadecyl ester	$C_{25}H_{42}O_2$	1610.92	0.025
	3-Isopropoxy-1,1,1,7,7,7-hexamethyl-3,5,5-tris(trimethylsiloxy) tetrasiloxane	$C_{18}H_{52}O_7Si_7$	1613	0.111
	Semicarbazide	CH_5N_3O	1615.36	0.001
	1,2-Propanediamine	$C_3H_{10}N_2$	1625.08	0.001
	Carbonic acid, bis(2-ethylhexyl) ester	$C_{17}H_{34}O_3$	1656.5	0.01
	Benzoic acid, hexyl ester	$C_{13}H_{18}O_2$	1665.64	0.011

	Semicarbazide	CH_5N_3O	1676.88	0.002
	Nickel tetracarbonyl	C_4NiO_4	1679.45	0.001
	2-(2,2-Dimethyl-propionyl)-1-(hydroxy-phenyl-methyl)-1,2,3,4-tetrahydroisoquinoline-3-carboxylic acid	$C_{22}H_{25}NO_4$	1680.59	0.017
	Heptadecane, 2,6,10,15-tetramethyl-	$C_{21}H_{44}$	1712.86	0.043
	Hexanoic acid, 2-ethyl-, anhydride	$C_{16}H_{30}O_3$	1717.27	0.028
	Heptadecane, 2,6,10,15-tetramethyl-	$C_{21}H_{44}$	1732.3	0.912
	Nickel tetracarbonyl	C_4NiO_4	1754.93	0.002
	Cyclononasiloxane, octadecamethyl-	$C_{18}H_{54}O_9Si_9$	1757.19	0.114
	N-(4-Nitrophenyl)-2-pyrrolidinecarboxamide, N'-acetyl	$C_{13}H_{15}N_3O_4$	1758.46	0.008
	Semicarbazide	CH_5N_3O	1776.52	0.002
	L-Prolinamide	$C_5H_{10}N_2O$	1786.44	0.004
	Semicarbazide	CH_5N_3O	1796.76	0.001
	Nickel tetracarbonyl	C_4NiO_4	1800.03	0
	3-Methylhexan-2-amine	$C_7H_{17}N$	1800.99	0.001
	Hydrazinecarboxamide	CH_5N_3O	1809.92	0.001
	Semicarbazide	CH_5N_3O	1829.39	0.002
	Squalene	$C_{30}H_{50}$	1835.7	0.13
	3-Isopropoxy-1,1,1,7,7,7-hexamethyl-3,5,5-tris(trimethylsiloxy) tetrasiloxane	$C_{18}H_{52}O_7Si_7$	1839.53	0.104
	Hydrazinecarboxamide	CH_5N_3O	1843.67	0.057
	Hydrazinecarboxamide	CH_5N_3O	1896.43	0.005
	1,2-Propanediamine	$C_3H_{10}N_2$	1910.64	0.002
	dl-Alanyl-l-alanine	$C_6H_{12}N_2O_3$	1915.17	0.001
	Hydrazinecarboxamide	CH_5N_3O	1936.29	0.004
	Semicarbazide	CH_5N_3O	1958.34	0.002

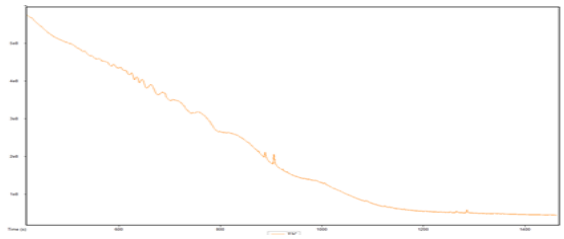

Figure 2: Spectrum of GCMS analysis and their volatile compounds representation of *P. australis* leaf extract.

Anti-oxidant activity

Table 04 shows the IC_{50} values of the anti-oxidant activity of leaves aqueous extract in comparison with ascorbic acid for DPPH (2,2-Diphenyl-1-picrylhydrazyl) and FRAP (ferric reducing antioxidant power) assays respectively.

Table 4: IC_{50} values of *P. australis* leaf extract and ascorbic acid according to DPPH and FRAP assays.

Test	Leaves aqueous extract	Ascorbic acid
	IC_{50} µg/ml	IC_{50} µg/ml
DPPH	1.339	0.033
FRAP	0.201	0.035

Anti-inflammatory activity

Figure 3 shows the IC_{50} levels of protein denaturation assay using BSA and leaves aqueous extract where diclofenac is used as a standard.

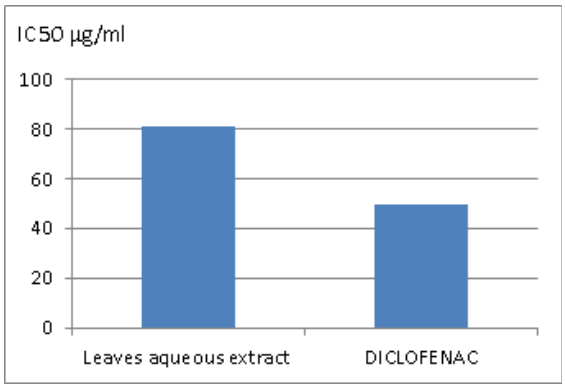

Figure 3: IC_{50} levels of protein denaturation assay using *P. australis* leaves aqueous extract in comparison with diclofenac

Hemolysis test

Figure 4 shows the results of red blood cell hemolysis in the presence of *P. australis* leaves aqueous extract, diclofenac is used as a standard.

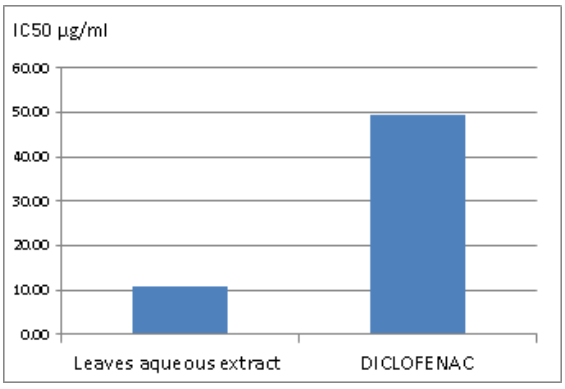

Figure 4: IC_{50} levels of red blood cells hemolysis assay using *P. australis* leaves aqueous extract in comparison with diclofenac

Discussion

Numerous bioactive components, including phenolics, terpenoids, tanins, alkaloides, carbohydrates, and flavonoids, were identified in *P. australis* leaves aqueous extract after phytochemical screening. The phenolic compounds are known to be antioxidants that neutralize free radicals, which are triggers of oxidative damage (9, 19, 20). Those secondary metabolites have played a major role in preventing and treating a variety of diseases including cancer, inflammation-related illnesses, diabetes, osteoporosis, cardiovascular disease, and neuro-degenerative diseases (21). Terpenoids have anticancer, anti-inflammatory, antibacterial, antiviral, and antimalarial properties, as well as the ability to enhance transdermal absorption, prevent and cure cardiovascular disease, and have hypoglycemic properties. Furthermore, earlier research has discovered that terpenoids have a wide range of potential uses, including insect resistance, immunoregulation, antioxidation, antiaging, and neuroprotection (22). As for the total phenolic content and the total flavonoids of *P. australis* leaves aqueous extract, it was 70,74±1,94 mg GA eq/ g dry extract and 3,64±0,98 mg Q eq/ g dry extract respectively (Table 02). The presence of hydroxyl groups (-OH) in the structure of phenolic compounds and flavonoids is responsible for free radical scavenging and functioning as antioxidants (19). The richness of *P. australis* leaves aqueous extract of different bioactive compounds and their variable application justify its wide use in traditional medicine. GC-MS analysis indicated that more than 200 components were identified in *P. australis* leaf extract which is dominated by 1-Dodecanol, Pyrazine, tetramethyl, Heptadecane, 2,6,10,15-tetramethyl, Cyclopentasiloxane, decamethyl, and Cyclotetrasiloxane, octamethyl.1-Dodecanol also known as lauryl alcohol is an organic fatty alcohol (23). Studies have shown that 1-dodecanol has antibacterial activity against S. aureus with a MIC of 6.25 µg/ml (24). It is also shown to be an antimycobacterial agent (25). Pyrazine, tetramethyl also called ligustrazine, is an organic volatile compound that many studies have been conducted to investigate its medical usefulness, including the decrease of platelet

aggregation, renal ischemia/reperfusion damage, and the prevention of atherosclerosis (26). Another study revealed its antioxidant activity (27). Tetramethylpyrazine's effects on improving postoperative adhesion of tissues have been reported in pharmacological investigations and therapeutic applications (28). Heptadecane, 2,6,10,15-tetramethyl is a compound that has demonstrated antituberculous activity (29) and anti-inflammatory activity (30). As for cyclopentasiloxane, decamethyl and cyclotetrasiloxane, octamethyl, they are well-known antimicrobial substances derived from many plant species (31, 32).

Regarding the antioxidant activity done by DPPH assay, it is increased when DPPH interacts with an antioxidant in the tested sample that might supply hydrogen. The color goes from deep violet to pale yellow (5). The potential of scavening free radicals is expressed by IC_{50} values which is the extract concentration capable of blocking 50% of DPPH free radicals (33), the lower is IC_{50} value, the higher the antioxidant power and vice versa (19). FRAP test is about reducing power which is related to a compound's capacity to transfer electrons, it can be used to anticipate its future antioxidant activity. This is due to the extract's polyphenols' predisposition to transfer electrons (34). Talking about anti-inflammatory activity, inflammation is a complicated pathological process that is a protective reaction of live tissue with the vascular system to inflammatory factor harm (35). The most significant drawback of the currently available powerful synthetic anti-inflammatory medications is their toxicity and recurrence of symptoms after withdrawal. As a result, individuals are going back to natural goods in the hope of finding safety and security (36). The aim of using plant extract is to increase the chances of having a synergic effect on all its components, otherwise, it might disappear when using each component on its own. Several pharmaceutical studies, including those for anti-inflammatory efficacy, revealed this (37). Results of the anti-inflammatory tests presented in Figure 02 and Figure 03 expressed by the IC_{50} levels reveal a distinguished anti-inflammatory effect, *P. australis* leaves aqueous extract has protected red blood cells from hemolysis effectively (IC_{50} = 10,73 µg/ml). Our results are consistent with Zhu *et al.* who deduced that *P. australis* the crude extract has strongly repressed macrophages responsible for inflammation (8).

Conclusion

Phytochemical screening results, GC-MS analysis results, and *in vitro* biological activities study validate the abundance of bioactive compounds in *P. australis* leaves aqueous extract that places it in a position to be a potential source of natural antioxidant and anti-inflammation bio-molecules.

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Conflict of interests: None declared.

References

1. Asif M. Chemistry and antioxidant activity of plants containing some phenolic compounds. Chemistry international. 2015; 1(1): 35-52.

2. Chetehouna S, Derouiche S, Reggami Y, Boulaares I. Sonchus maritimus Extract-Loaded Niosomes Bioconjugated by Linoleic Acid in Hepatic Encephalopathy Induced by High-Fructose Diet in Albino Wistar Rats. Archives of Razi Institute. 2024;79(1):194-205.

3. Oladipo AO, Iku SI, Ntwasa M, Nkambule TT, Mamba BB, Msagati TA. Doxorubicin conjugated hydrophilic AuPt bimetallic nanoparticles fabricated from Phragmites australis: Characterization and cytotoxic activity against human cancer cells. Journal of Drug Delivery Science and Technol-

ogy. 2020; 57(101749.

4. Chen Y, Li L, Jiang L-R, Tan J-Y, Guo L-N, Wang X-L, Dong W, Wang W-B, Sun J-K, Song B. Alkaloids constituents from the roots of Phragmites australis (Cav.) Trin. ex Steud. with their cytotoxic activities. Natural Product Research. 2022; 36(6): 1454-1459.

5. Sohaib M, Al-Barakah FN, Migdadi HM, Husain FM. Comparative study among Avicennia marina, Phragmites australis, and Moringa oleifera based ethanolic-extracts for their antimicrobial, antioxidant, and cytotoxic activities. Saudi Journal of Biological Sciences. 2022; 29(1): 111-122.

6. Chen S, Ju M, Luo Y, Chen Z, Zhao C, Zhou Y, Fu J. Hepatoprotective and antioxidant activities of the aqueous extract from the rhizome of Phragmites australis. Zeitschrift für Naturforschung C. 2013; 68(11-12): 439-444.

7. Sim M-O, Ham JR, Lee M-K. Young leaves of reed (Phragmites communis) suppress melanogenesis and oxidative stress in B16F10 melanoma cells. Biomedicine & Pharmacotherapy. 2017; 93(165-171.

8. Zhu L, Zhang D, Yuan C, Ding X, Shang Y, Jiang Y, Zhu G. Anti-Inflammatory and antiviral effects of water-soluble crude extract from Phragmites australis in vitro. Pakistan journal of pharmaceutical sciences. 2017; 30(4): 1357-1362.

9. Derouiche S, Azzi M, Hamida A. Phytochemical analysis and antioxidant property of rhizome extracts aqueous of Phragmites australis in alloxan diabetic rats. Asian Journal of Pharmacy and Technology. 2019; 9(4): 249.

10. Wadood A, Ghufran M, Jamal SB, Naeem M, Khan A, Ghaffar R. Phytochemical analysis of medicinal plants occurring in local area of Mardan. Biochem anal biochem. 2013; 2(4): 1-4.

11. Harborne A: **Phytochemical methods a guide to modern techniques of plant analysis**: springer science & business media; 1998.

12. Harborne JB: **A guide to modern techniques of plant analysis**: Chapman and hall; 1973.

13. Boizot N, Charpentier J-P. Méthode rapide d'évaluation du contenu en composés phénoliques des organes d'un arbre forestier. Le Cahier des Techniques de l'INRA, In: Numéro spécial. 2006; 79-82.

14. Dehpour AA, Ebrahimzadeh MA, Fazel NS, Mohammad NS. Antioxidant activity of the methanol extract of Ferula assafoetida and its essential oil composition. Grasas y Aceites. 2009; 60(4): 405-412.

15. Nwidu LL, Elmorsy E, Aprioku JS, Siminialayi I, Carter WG. In vitro anti-cholinesterase and antioxidant activity of extracts of Moringa oleifera plants from Rivers State, Niger Delta, Nigeria. Medicines. 2018; 5(3): 71.

16. Oyaizu M. Studies on products of browning reaction antioxidative activities of products of browning reaction prepared from glucosamine. The Japanese journal of nutrition and dietetics. 1986; 44(6): 307-315.

17. Vennila K, Chitra L, Balagurunathan R, Palvannan T. Comparison of biological activities of selenium and silver nanoparticles attached with bioactive phytoconstituents: green synthesized using Spermacoce hispida extract. Advances in Natural Sciences: Nanoscience and Nanotechnology. 2018; 9(1): 015005.

18. Vinjamuri S, Afshan S, Shekar S, Saraswathi V. Evaluation of hemolytic activity,

ATPase inhibitory activity and antitumor activity of TLC extract of lemon grass (Cymbpogon citratus). International Journal of Pharmacognosy and Phytochemical Research. 2015; 7(4): 785-788.

19. Chetehouna S, Derouiche S, Atoussi O. Identification of New Alkaloids from Algerian Purslane by HPLC-QTOF-MS and Beneficial Effect of Purslane Enriched with Zinc on Experimental Alzheimer Disease in Rats. Current Trends in Biotechnology and Pharmacy 2024; 18(1): 1595-1607.

20. Boulaares I, Derouiche S, Niemann J. HPLC-Q-TOF-MS analysis of phenolic compounds, in vitro biological activities and in vivo acute toxicity evaluation of Ocimum Basilicum L. Fresenius Environmental Bulletin. 2024; 33(2): 73-82

21. Chetehouna S, Derouiche S, Boulaares I, Réggami Y. Phytochemical profile, anti-inflammatory analysis and cytotoxic activity of SmE-SeNPs against breast (MCF-7) cancer cells. Biocatalysis and Agricultural Biotechnology. 2024; 57: 103122.

22. Yang W, Chen X, Li Y, Guo S, Wang Z, Yu X. Advances in pharmacological activities of terpenoids. Natural Product Communications. 2020; 15(3): 1934578X20903555.

23. Chetehouna S, Derouiche S, Réggami Y, Boulaares Islam , Frahtia A. Gas Chromatography Analysis, Mineral Contents and Anti-inflammatory Activity of Sonchus maritimus. Tropical Journal of Natural Product Research, 2024; 8(4):6787-6798

24. Chatterjee S, Karmakar A, Azmi SA, Barik A. Antibacterial activity of long-chain primary alcohols from Solena amplexicaulis leaves. Proceedings of the Zoological Society. 2017; 71(313-319.

25. Mukherjee K, Tribedi P, Mukhopadhyay B, Sil AK. Antibacterial activity of long-chain fatty alcohols against mycobacteria. FEMS microbiology letters. 2013; 338(2): 177-183.

26. Feng L, Ke N, Cheng F, Guo Y, Li S, Li Q, Li Y. The protective mechanism of ligustrazine against renal ischemia/reperfusion injury. Journal of Surgical Research. 2011; 166(2): 298-305.

27. Wang A, Zhang J, Li Z. Correlation of volatile and nonvolatile components with the total antioxidant capacity of tartary buckwheat vinegar: Influence of the thermal processing. Food research international. 2012; 49(1): 65-71.

28. Yan S, Yue Y-z, Zong Y, Zeng L. Tetramethylpyrazine improves postoperative tissue adhesion: a drug repurposing. Chinese journal of integrative medicine. 2019; 25(7): 554-560.

29. Poongulali S, Sundararaman M. Antimycobacterial, anticandidal and antioxidant properties of Terminalia catappa and analysis of their bioactive chemicals. International Journal of Pharmacy and Biological Sciences. 2016; 6(2): 69-83.

30. Naik NM, Krishnaveni M, Mahadevswamy M, Bheemanna M, Nidoni U, Kumar V, Tejashri K. Comparative analysis of supercritical fluid and Soxhlet Prosopis juliflora leaves extract with antifungal traits. Research Square. 2022; 1-15.

31. Keskın D, Ceyhan N, Uğur A, Dbeys AD. Antimicrobial activity and chemical constitutions of West Anatolian olive (Olea europaea L.) leaves. Journal of Food, Agriculture & Environment. 2012; 10(2): 99-102.

32. Rasyid A, Putra MY. Antibacterial and antioxidant activity of sea cucumber extracts collected from Lampung waters, Indonesia. Kuwait Journal of Science. 2023; 50(4): 615-621.

33. Boulaares Islam, Derouiche Samir, Guemari Imane Yousra. Protective effect of ObE against Doxorubicin-Induced immunosuppression and Cardiotoxicity in Rats. Research Journal of Pharmacy and Technology. 2024; 17(4):1839-1843.

34. Derouiche S, Chetehouna S, Atoussi W. "The Effects of aqueous leaf extract of Portulaca oleracea on haemato-biochemical and histopathological changes induced by Sub-chronic Aluminium toxicity in male wistar rats." Pharmacological Research-Modern Chinese Medicine. 2022; 100101.

35. Zhou R, Cui M, Wang Y, Zhang M, Li F, Liu K. Isolation, structure identification and anti-inflammatory activity of a polysaccharide from Phragmites rhizoma. International journal of biological macromolecules. 2020; 161(810-817.

36. Banerjee S, Chanda A, Adhikari A, Das A, Biswas S. Evaluation of phytochemical screening and anti inflammatory activity of leaves and stem of Mikania scandens (L.) wild. Annals of medical and health sciences research. 2014; 4(4): 532-536.

37. Atoussi O , Chetehouna S, Derouiche S. Biological properties and Acute Toxicity Study of Copper oxide nanoparticles prepared by aqueous leaves extract of Portulaca oleracea (L). Asian Journal of Pharmaceutical Research. 2020; 10(2): 89-94.

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Evaluation of the Free Radical Scavenging Activities and Antibacterial Activities of the Extracts of *Lindernia ruellioides* (Colsmann) Pennell.

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Abstract

Around the world, plants have been used as medicine to ameliorate a broad spectrum of ailments, leveraging their wealth of phytochemicals. Due to their availability and reasonably priced, plant-based medicines have occasionally supplanted synthetic pharmaceuticals. *Lindernia ruellioides,* locally called as "Thasuih" in Mizoram has been used by indigenous practitioners and traditional healers for treating various ailments. However, there is no scientific validation for this traditional use. Scientific validation at known doses may provide information about its safety and efficacy. Therefore, the present study endeavours to estimate the phytochemical contents and determine the free radical scavenging activity and antibacterial activity of various extracts of The results were evaluated statistically using SPSS (Online) and Graph pad prism (Online).The plant was washed and allowed to shade dried at room temperature and then powdered. It was then subjected to sequential cold maceration using different solvents such as petroleum ether, chloroform, ethanol and distilled water. Phytochemical analysis was carried out using standard procedures to identify the constituents. The ability of the extracts to inhibit the generation of various free radicals was determined by assessing the scavenging activity of 1,1-diphenyl-2-picrylhydrazyl radicals (DPPH), 2, 2'-azino-bis-3-ethylbenzothiazoline-6-sulfonic acid (ABTS) and superoxide radicals. Antibacterial activity was determined using disc diffusion method and minimum inhibitory concentration. The results were evaluated statistically using SPSS (Online) and Graph pad prism (Online). The preliminary phytochemical screening disclosed that the plant incorporates an assortment of phytochemicals such as alkaloids, saponins, flavonoids, tannins, steroids, glycosides, phlobatannins and terpenoids. Among the various extracts of *L. ruellioides*, ethanol extract has the highest total phenolic (327.97 ± 1.77 mg GAE/g of dry extract) and flavonoid contents (264.95 ± 0.71 mg quercetin equivalent/g of dry extract). Different extracts of *L. ruellioides* when analysed for their scavenging activities showed significant inhibition of DPPH, ABTS and superoxide in a concentration dependent manner. The ethanolic extract showed the highest scavenging activities for DPPH and ABTS with IC_{50} 158.0 ± 4.82 µg/ml and 112.9 ± 6.47 µg/ml respectively. However, aqueous extract was found to possess the highest scavenging activity for superoxide with IC_{50} 135.5 ± 5.02 µg/ml. The different extracts were also found to be active against the test microorganisms, thus, justifying the folkloric use of the plant.

Keywords: *Lindernia ruellioides,* free radical scavenging, antibacterial, disc diffusion, minimum inhibitory concentration.

Introduction

During normal metabolic process, free radicals are normally generated in our body. Reactive oxygen species (ROS) as well as reactive nitrogen species (RNS) are generally the by-products as a result of the cellular redox process. They play a dual role as both detrimental and beneficial effects (1). At high concentrations, they are highly reactive and toxic and cause damage to cell membrane, proteins, lipids and deoxyribonucleic acid (DNA) which leads to oxidative stress. This oxidative stress cause tissue damage resulting in several diseases such as neurodegenerative disease, cardiovascular disease, diabetes, cancer, aging, rheumatoid arthritis and cataract (2-4). Antioxidant play a key role in protecting our body against free radical damage. They balance free radical production and detoxify them when in excess (5-7).

Regardless of the extensive use as traditional medicine, scientific validation is still limited. *L. ruellioides,* belonging to the family Linderniaceae is an erect annual herb found commonly in semi-shaded areas (8). Traditionally, it has been used for dressing cuts, wounds, bruises, boils, jaundice, snakebite, dysentery, urinary trouble and quick healing of wounds when applied externally and the juices of the leaves are also used for massaging on strains (9,10). Regardless of the extensive use as traditional medicine, scientific validation is still limited.

Materials and Methods

Collection of plant material and preparation of extracts

L. ruellioides was collected from Reiek, Aizawl district, Mizoram, India (23.678ºN, 92.603ºE) during the months of June-August. Identification and authentication of the plant was done by Natural History Museum Mizoram, Mizoram University with accession no. NHMM-P/000160. *L. ruellioides* was washed and allowed to shade dried at room temperature and then powdered. It was then subjected to sequential cold maceration using different solvents such as petroleum ether, chloroform, ethanol and distilled water. The liquid extracts were then filtered and evaporated to dryness in an oven and was stored at 4°C for further use. Hereafter, the chloroform, ethanolic and aqueous extracts of *L. ruellioides* will be called as LRCE, LREE and LRAE respectively.

Phytochemical screening

Different extracts of *L. ruellioides* were analysed for the presence of alkaloids, saponins, flavonoids, tannins, steroids, glycosides, phlobatannins and terpenoids using standard protocol (11,12).

Quantitative analysis

Determination of total phenolic content (13)

5 ml of Folin-Ciocalteau's reagent (diluted ten-fold) was mixed with 1 ml of *L. ruellioides* extract, dissolved in their respective solvent, at the concentration ranging from 0.25-8.0 mg/ml. Sodium carbonate (4 ml, 0.115 mg/ml) was added to the mixture after 5 mins of incubation at room temperature. Then the mixture was incubated at room temperature for 2 hrs followed by measuring the absorbance at 765 nm using UV-Visible spectrophotometer. Calibration curve was also prepared by mixing methanolic solution of gallic acid (1 ml) with the reagents. All determinants were carried out in triplicate. The total phenolic content in each extract were expressed as gallic acid equivalents (GAE) mg/ml of the dry extract.

Determination of total flavonoids content (14)

0.25 ml of extract (0.25-8.0 mg/ml; dissolved in the appropriate solvent) and quercetin standard solution was mixed with 1.25 ml of distilled water followed by the addition of 75 µl of 5% (w/v) sodium nitrite solution. After few minutes, 150 µl of 10% (w/v) aluminium chloride solution was added and allowed to stand for further 5 mins before the addition of 0.5 ml of 1M sodium hydroxide. The mixture was then diluted with distilled water to a volume of 2.5 ml. At 510

nm, absorbance was immediately measured. Quercetin equivalents were used to represent the result (mg/g extract).

In vitro antioxidant assays

DPPH radical scavenging activity (15)

To different concentrations of various extracts of *L. ruellioides* (0.5 ml, 20-1000 µg/ml), 1 ml of methanolic solution of 0.1 M DPPH was added. The mixture was then allowed to stand in the dark for 30 mins and absorbance was measured at 523 nm. Methanol was utilized as the baseline correction. The results were compared with control prepared as above without sample. The antioxidant activity of the extract was expressed as IC_{50}, the concentration (µg/ml) of extract that inhibited 50% of DPPH radicals. Ascorbic acid was used as the standard and each study was performed in triplicate. The amount of scavenging was then calculated using the formula:

% scavenging = [(Ablank - Asample)/Ablank] × 100

where A_{blank} the is absorbance of the control reaction and A_{sample} is the absorbance of the solution containing the plant extract.

ABTS radical scavenging activity (16)

Equal volume of 2.45 mM potassium persulfate solution and a 7 mM ABTS solution were combined to create a stock solution. The solution was incubated at room temperature in the dark for 12 hrs to yield a dark-coloured solution containing $ABTS^{\cdot+}$ radicals. A working solution was prepared freshly before each assay by diluting the stock solution with 50% methanol for an initial absorbance of about 0.700 (± 0.02) at 745 nm. The scavenging activity was then evaluated by combining 150 µl of various extract fractions (20-1000 µg/ml dissolved in their respective solvents) with 1.5 ml of ABTS working standard. At 745 nm, the drop in absorbance was measured. Ascorbic acid was used as positive control. The scavenging activity was then estimated based on the formula:

% scavenging = [(Ablank - Asample)/Ablank] × 100where A_{blank} is the absorbance of the control reaction A_{sample} is the absorbance of the solution containing the plant extract.

Superoxide ($O_2^{\cdot-}$) radical scavenging activity (17)

To the reaction mixture containing 0.2 ml of nitroblue tetrazolium (1 mg/ml in dimethyl sulfoxide) and 0.6 ml of extract (20-1000 µg/ml), 2 ml of alkaline dimethyl sulfoxide (DMSO) (1 ml DMSO in 5 mM sodium hydroxide) was included to provide a complete volume of 2.8 ml. The absorbance was recorded at 560 nm. The blank consisted of pure DMSO instead of alkaline DMSO. Ascorbic acid was used as the standard and the ability of *L. ruellioides* extracts to scavenge the superoxide radical was calculated using the formula:

% scavenging = $(A_e - A_o / A_e) \times 100$

where Ablank is the absorbance of the control reaction and Asample is the absorbance of the solution containing the plant extract.Antibacterial activity

The antibacterial activity was assessed against three bacteria species: *Escherichia coli* (MTCC-40), *Bacillus subtilis* (MTCC-121) and *Klebsiella pneumoniae* (MTCC-39).

Disc diffusion method (18)

Agar plates were prepared which were inoculated with the test microorganisms. Then the paper disc containing two different concentrations (20 mg and 10 mg) of plant extract, 25 µg of streptomycin disc (standard) and paper disc (control) that contain 5% DMSO for LRCE and distilled water for LREE and LRAE were kept carefully on the surface of the prepared agar plate. Then, the plates were incubated for 24 hrs at 37°C in inverted position. After incubation, the extract possessing those activity was taken for measuring the zone of inhibition and compared with the standard antibiotic.

Minimum inhibitory concentration (MIC) (19)

Agar plates were prepared which were inoculated with the test microorganisms. Following that, the paper discs with six different concentrations (10 mg, 5 mg, 2.5 mg, 1.25 mg, 0.625 mg and 0.3125 mg) of plant extract and 25 µg streptomycin disc were carefully placed on the agar plate's surface. Negative control was not kept as 5% DMSO and distilled water does not show any antimicrobial activity in the previous disc diffusion experiment. The plates were then incubated for 24 hrs inverted at 37°C. The lowest concentration inhibiting the growth of microorganism was noted and considered as MIC for each test microorganisms.

Statistical Analysis

Data are expressed as mean ± standard error of mean. One-way analysis of variance (ANOVA) was performed to test the significant variations followed by Tukey multiple comparison of means. *P* value of less than 0.05 was considered statistically significant. SPSS (Online) and Graph pad prism (Online) were used for statistical and graphical evaluations.

Results and Discussion

Qualitative phytochemical analysis

Qualitative phytochemical screening showed the presence of various naturally occurring compounds like alkaloids, saponins, flavonoids, tannins, phlobatannins and terpenoids in LREE. Alkaloids, flavonoids, tannins and steroids were found in LRAE. Alkaloids, saponins and tannins were also found to be present in LRCE (Table 1). These phytochemicals are secondary metabolites that contribute to flavour and colour (20) and have been reported to possess several pharmacological potentials which includes antioxidants (21), antimalarial (22), antimicrobial activities (23) and reduce the risk of many diseases (24).

Table 1: Qualitative phytochemical evaluation of different *L. ruellioides* extracts.

Phytochemicals	Reagent	Colour indication	LRCE	LREE	LRAE
Alkaloids	Dragendroff's reagent	Reddish brown precipitate	+	+	+
Saponins	Olive oil	Whitish emulsion	+	+	-
Flavonoids	Sulphuric acid, Magnesium turnings	Pink red color	-	+	+
Tannins	Ferric chloride	Brownish green or blue-black color	+	+	+
Steroids	Sulphuric acid	Red colour	-	-	+
Glycosides	Glacial acetic acid, Ferric chloride, Sulphuric acid	Brown ring	-	-	-
Phlobatannins	Hydrochloric acid	Red precipitate	-	+	-
Terpenoids	Sulphuric acid	Reddish brown	-	+	-

'+' sign denotes the presence of phytochemicals while '-' sign denotes the absence of phytochemicals. LRCE- *L. ruellioides* chloroform extract; LREE- *L. ruellioides* ethanolic extract; LRAE- *L. ruellioides* aqueous extract.

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Determination of Total phenolic and flavonoid content:

The total phenolic and total flavonoid content of *L. ruellioides* extracts increased in a concentration dependent manner (Figure 1 & 2). At 8 mg/ml, LREE has significantly higher (*P* 0.001) total phenolic content (327.97 ± 1.77 mg GAE /g of dry extract) than LRAE (209.63 ± 0.69 mg GAE /g of dry extract) and LRCE (196.51 ± 1.71 mg GAE /g of dry extract). For total flavonoid, LREE also has significantly higher (*P* 0.001) content (264.95 ± 0.71 mg quercetin equivalent/g of dry extract) than LRAE (217.44 ± 0.15 mg quercetin equivalent/g of dry extract) and LRCE (210.59 ± 0.41 mg quercetin equivalent/g of dry extract). Phenolic compounds have been reported to show antioxidant activity by scavenging or stabilizing free radicals due to their conjugated ring structures and presence of hydroxyl groups (25) and have been reported to exhibit antiallergenic, antimicrobial, anti-inflammatory and cardioprotective effects (26). Similarly, flavonoids are also reported to have antioxidative action and reduce their formation by chelating the metals (27). In addition, there have been reports of flavonoids to serve as health-promoting compound and protect against several diseases (28).

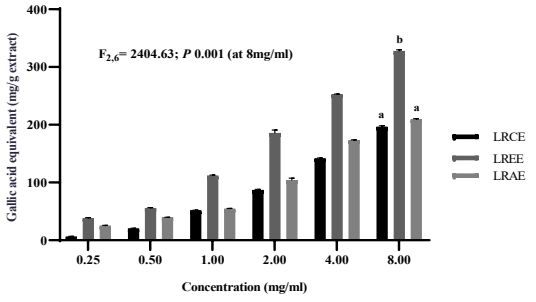

Fig. 1: Phenolic content of *L. ruellioides* extracts determined as Gallic acid equivalent. LRCE- *L. ruellioides* chloroform extract; LREE- *L. ruellioides* ethanol extract; LRAE- *L. ruellioides* aqueous extract. Values are expressed as Mean ± SEM, n=3. Different letters indicate significant variation.

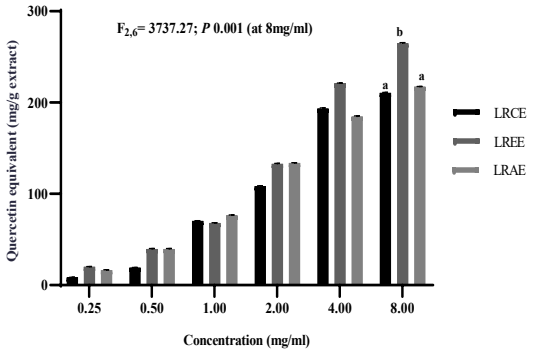

Fig. 2: Flavonoid content of various extracts of *L. ruellioides* determined as Quercetin equivalent. LRCE- *L. ruellioides* chloroform extract; LREE- *L. ruellioides* ethanol extract; LRAE- *L. ruellioides* aqueous extract. Values are expressed as Mean ± SEM, n=3. Different letters indicate significant variation.

In vitro antioxidant assay

DPPH radical scavenging activity

L. ruellioides scavenged DPPH radicals in a concentration dependent fashion as indicated by the discolouration of DPPH. The maximum activity of LREE, LRCE and LRAE to scavenged DPPH was noted at 500 µg/ml, 600 µg/ml and 900 µg/ml respectively. LREE was most potent as it effectively inhibited DPPH radical formation and showed the highest scavenging activity (IC_{50} 158.0 ± 4.82 µg/ml) followed by LRAE (IC_{50} 276.4 ± 6.32 µg/ml) and the lowest scavenger was LRCE (IC_{50} 330.43 ± 5.34 µg/ml). IC_{50} of all the extracts are statistically significant when compared to the standard ascorbic acid (IC_{50} 12.47 ± 0.13 µg/ml). Different extracts of various plants have been shown to inhibit the generation of DPPH free radicals earlier (29-34). Compounds such as cysteine, glutathione, ascorbic acid, tocopherol, poly-hydroxyl aromatic compounds have been known to have the ability to reduce DPPH by hydrogenation (35,36).

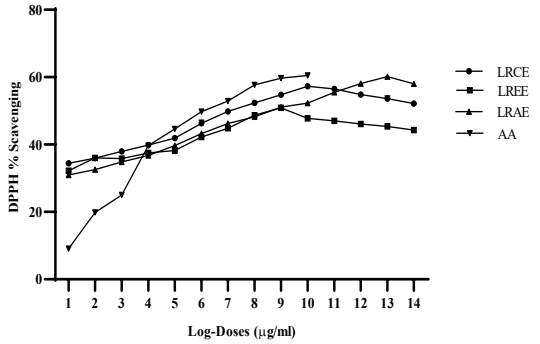

Fig. 3: DPPH radical scavenging activity of several extracts of *L. ruellioides* and standard ascorbic acid. LRCE- *L. ruellioides* chloroform extract; LREE- *L. ruellioides* ethanol extract; LRAE- *L. ruellioides* aqueous extract; AA- Ascorbic acid. Values are expressed as mean ± SEM, n=3.

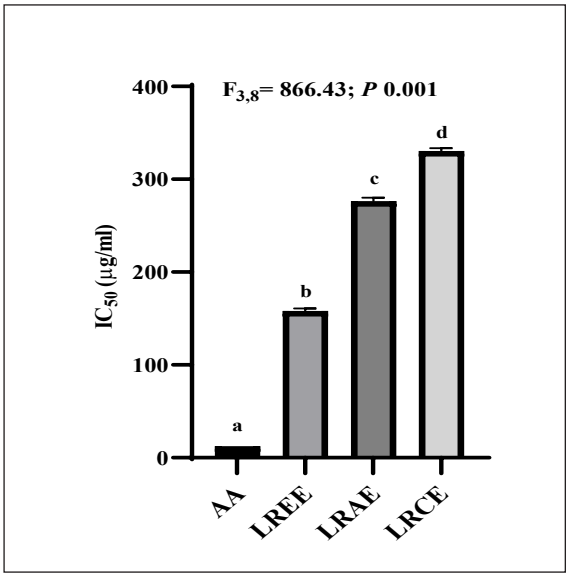

Fig. 4: IC_{50} (µg/ml) for DPPH. AA- Ascorbic acid. LRCE- *L. ruellioides* chloroform extract; LREE- *L. ruellioides* ethanol extract; LRAE- *L. ruellioides* aqueous extract. Values are expressed as Mean ± SEM, n=3. Different letters indicate significant variation.

ABTS radical scavenging activity

L. ruellioides also showed a concentration dependent increase in the scavenging of ABTS radical. The maximum scavenging activity was observed at 200 µg/ml for both LREE and LRAE and 500 µg/ml for LRCE. LREE scavenged the ABTS radicals more efficiently as it showed the highest scavenging activity (IC_{50} 112.9 ± 6.47 µg/ml), followed by LRAE (IC_{50} 222.33 ± 3.18 µg/ml). LRCE was least effective in neutralizing the ABTS (IC_{50} 318 ± 12.19 µg/ml). IC_{50} of all the extracts were statistically significant when compared to the standard ascorbic acid (IC_{50} 13.52 ± 0.25 µg/ml). Some earlier studies had reported a resembling effect by using different plant extracts (37,38). Stable free radical ABTS is produced when a potent oxidizing agent reacts with another substance with the ABTS salt. A dark-coloured solution's reduction by an antioxidant that donates hydrogen is assessed by its distinctive long-wave (745 nm) absorption spectra (39). The level of decolourization indicates the effective inhibition of the $ABTS^{\cdot+}$ (40). The $ABTS^{\cdot+}$ scavenging activity could be due to high phenolic contents.

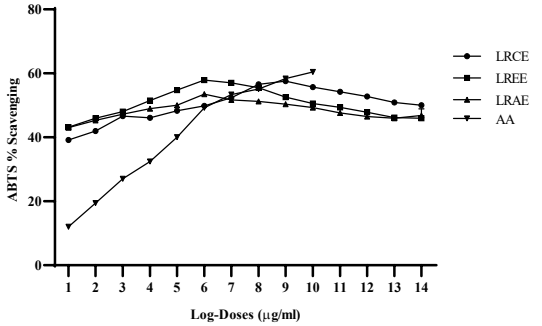

Fig. 5: ABTS radical scavenging activity of various extracts of *L. ruellioides* and standard ascorbic acid. LRCE- *L. ruellioides* chloroform extract; LREE- *L. ruellioides* ethanol extract; LRAE- *L. ruellioides* aqueous extract; AA-Ascorbic acid. Values are expressed as mean ± SEM, n=3.

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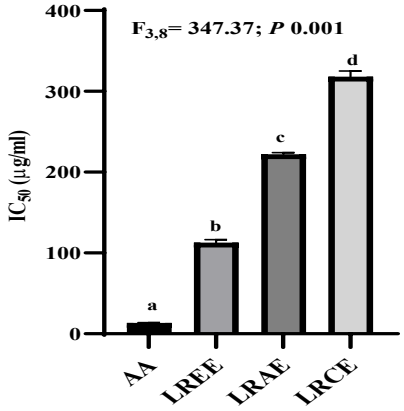

Fig. 6: IC_{50} (µg/ml) for ABTS. AA- Ascorbic acid; LRCE- *L. ruellioides* chloroform extract; LREE- *L. ruellioides* ethanol extract; LRAE- *L. ruellioides* aqueous extract. Values are expressed as Mean ± SEM, n=3. Different letters indicate significant variation.

Superoxide radical ($O_2^{\bullet-}$) scavenging activity

Various extract of *L. ruellioides* inhibit the generation of superoxide radicals in a concentration dependent manner. LRAE scavenged the $O_2^{\bullet-}$ most effectively with a peak scavenging activity at 600 µg/ml (IC_{50} 135.5 ± 5.02 µg/ml). For LREE and LRCE peak scavenging activity occurred at 700 µg/ml (IC_{50} 214.9 ± 3.59 µg/ml) and 1000 µg/ml (IC_{50} 366.43 ± 6.57 µg/ml) respectively. The standard ascorbic acid had an IC_{50} 15.18 ± 0.14 µg/ml for $O_2^{\bullet-}$. A number of plant extracts and some compound plant formulations were found to inactivate the formation of $O_2^{\bullet-}$ radicals in a dose dependent manner (31,33,34,38,41). Neutralization of superoxide radical is necessary to protect the cells from oxidative stress (42). It has also been reported that antioxidant properties of some flavonoids are effective mainly through scavenging superoxide radical (43). Thus, the presence of flavonoids in *L. ruellioides* might be responsible for their scavenging activity.

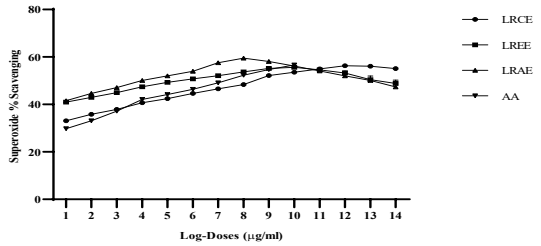

Fig. 7: Superoxide radical scavenging activity of various extracts of *L. ruellioides* and standard ascorbic acid. LRCE- *L. ruellioides* chloroform extract; LREE- *L. ruellioides* ethanol extract; LRAE- *L. ruellioides* aqueous extract; AA- Ascorbic acid. Values are expressed as Mean ± SEM, n=3.

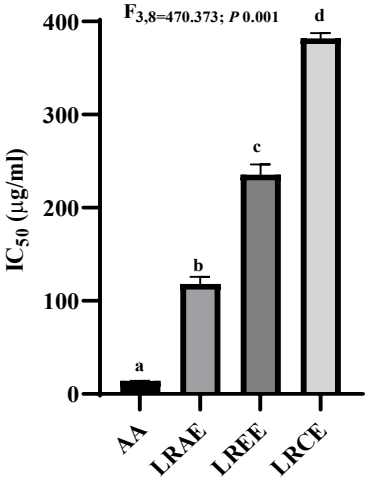

Fig. 8: IC_{50} (µg/ml) for superoxide radical. AA- Ascorbic acid; LRCE- *L. ruellioides* chloroform extract; LREE- *L. ruellioides* ethanol extract; LRAE- *L. ruellioides* aqueous extract. Values are expressed as Mean ± SEM, n=3. Different letters indicate significant variation.

Antimicrobial activity of plant extract

Disc diffusion method

The present study revealed that LRCE, LREE and LRAE were active against the test organisms as they show remarkable zone of

inhibition. At 10 mg/ml, LREE was most active against the three microorganisms with zone of inhibition of 7.6 ± 0.16 mm, 6.1 ± 0.16 mm and 7 ± 0.28 mm for *E. coli*, *B. subtilis* and *K. pneumoniae* respectively. However, at 20 mg/ml, LRCE was most effective with zone of inhibition of 9.3 ± 0.16 mm, 8.1 ± 0.16 mm and 9.1 ± 0.16 mm for *E. coli*, *B. subtilis* and *K. pneumoniae* respectively. Moreover, from the study, it is seen that 5% DMSO and distilled water, the solvents used for dissolving the extract does not show any antibacterial activity.

Table 2: Antimicrobial activity of LRCE, LREE, LRAE on different test microorganisms.

Extracts (10µg/disc)	Conc. (mg/ml)	Zone of inhibition (mm)*		
		E. coli	*B. subtilis*	*K. pneumoniae*
LRCE	10	7.1 ± 0.16	6.1 ± 0.16	6.5 ± 0.28
	20	9.3 ± 0.16	8.1 ± 0.16	9.1 ± 0.16
	Standard	17.5 ± 0.28	19.1 ± 0.16	17.3 ± 0.16
LREE	10	7.6 ± 0.16	6.1 ± 0.16	7 ± 0.28
	20	8.1 ± 0.16	7.1 ± 0.16	8.1 ± 0.16
	Standard	17.6 ± 0.16	19.6 ± 0.16	16.8 ± 0.16
LRAE	10	6.6 ± 0.33	5.3 ± 0.16	6.6 ± 0.16
	20	7.6 ± 0.16	6.1 ± 0.16	7.1 ± 0.16
	Standard	16.1 ± 0.16	16.1 ± 0.16	17.1 ± 0.16

* Indicate the zone of inhibition shown above is the mean of three readings and includes the diameter of the paper disc, i.e., 5 mm. LRCE- *L. ruellioides* chloroform extract; LREE- *L. ruellioides* ethanolic extract; LRAE- *L. ruellioides* aqueous extract.

MIC

The minimum concentration of the crude extract that inhibits the growth of the test microorganism was determined and recorded as MIC of the extract on that particular organism.

Table 3: Minimum inhibitory concentration (MIC) for different extracts of *L. ruellioides* on different test microorganisms.

Test organisms	MIC of LRCE	MIC of LREE	MIC of LRAE
Escherichia coli (MTCC- 40)	1.25 mg/ml	0.625mg/ml	2.5 mg/ml
Bacillus subtilis (MTCC- 121)	2.5 mg/ml	0.625mg/ml	5 mg/ml
Klebsiella pneumoniae (MTCC- 39)	0.625 mg/ml	1.25 mg/ml	5 mg/ml

MIC- Minimum inhibitory concentration; LRCE- *L. ruellioides* chloroform extract; LREE- *L. ruellioides* ethanol extract; LRAE- *L. ruellioides* aqueous extract.

Our result (Table 3) showed that LREE is quite active even at low concentrations with an MIC value of 0.625 mg/ml on *E. coli* and *B. subtilis* and 1.25 mg/ml on *K. pneumonia*. LRCE is also quite active on *E. coli* and *K. pneumonia* having MIC at 1.25 mg/ml and 2.5 mg/ml for *B. subtilis.* LRAE show MIC for *E. coli* at 2.5 mg/ml and for *B. subtilis* and *K. pneumonia* at 5 mg/ml. The presence of phytochemicals like flavonoids and tannins may be the cause of its antibacterial properties (44,45). These findings are in accordance with earlier studies (46-48). The above findings tell us that *L. ruellioides* has antibacterial activity even at low concentration,

however, isolation and purification of bioactive compounds responsible for the antibacterial activity need further investigation.

Conclusion

The results of phytochemical analysis validated the presence of various classes of bioactive chemical constituents. It also demonstrates that various extracts of *L. ruellioides* exhibit a concentration dependent inhibition against DPPH, ABTS and superoxide which might be due to presence of significant amounts of phenolic and flavonoid contents. Notably, its antibacterial activity is also quite remarkable against the test microorganisms. It may be concluded that the significant number of phytochemicals present in *L. ruellioides* attributed to their free radical scavenging activity and antibacterial property as well. Thus, the results of the present study substantiate the folkloric use of *L. ruellioides* urging in-depth exploration to unravel its mechanism of action.

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References

1. Sharma, U.S. and Kumar, A. (2011). *In vitro* antioxidant activity of *Rubus ellipticus* fruits. J Adv Pharm Technol Res, 2: 47-50.
2. Pham-Huy, L.A., He, H. and Pham-Huy, C. (2008). Free radicals, antioxidants in disease and Health. Int J Biomed Sci, 4: 89-96.
3. Bandyopadhyay, U., Das, D. and Banerjee, R.K. (1999). Reactive oxygen species: oxidative damage and pathogenesis. Current Science, 77: 658-666.
4. Sung, C.C., Hsu, Y.C., Chen, C.C., Lin, Y.F. and Wu, C.C. (2013). Oxidative stress and nucleic acid oxidation in patients with chronic kidney disease. Oxid Med Cell Longev, 2013.
5. Palaniswamy, R. and Padma, P.R. (2011). Free radical scavenging activity of *Majorana hortensis* leaves. Anc Sci Life, 30: 96-99.
6. Valko, M., Leibfritz, D., Moncol, J., Cronin, M.T.D., Mazur, M. and Telser, J. (2007). Free radicals and antioxidants in normal physiological functions and human disease. Int J Biochem Cell Biol, 39: 44-84.
7. Lobo, V., Patil, A., Phatak, A., and Chandra, N. (2010). Free radicals, antioxidants and functional foods: Impact on human health. Pramacogn Rev, 4: 118-126.
8. Cook, C.D.K. (1996). Aquatic and wetland plants of India: a reference book and identification manual for the vascular plants found in permanent or seasonal fresh water in the subcontinent of India South of the Himalayas. Oxford University Press, Oxford, New York.
9. Wei, J.C., Wang, P.C., Zhou, X.L., Tang, K., Luo, Q., Xu, Qin., Chen, XJ., Liang, C.Q. and Chen, X. (2017). The caffeoyl phenylethanoid glycosides from *Lindernia ruellioides* and their anti-HBV effects. J Asian Nat Prod Res, 20: 757-762.
10. Sikdar, M. and Dutta, U. (2008). Traditional Phytotherapy among the Nath People of Assam. Stud Ethno-med, 2: 39-45.
11. Trease, G.E. and Evans, W.C. (2002). Pharmacognosy (15th edition), Saunders Publishers, London, pp. 42-44.
12. Harborne, J.B. (1998). Phytochemical methods: A guide to modern techniques of plant analysis (2nd edition), Chapmann and Hall Publishers, London.

13. Singleton, V.L., Orthofer, R. and Lamuela-Raventos, R.M. (1999). Analysis of total phenols and other oxidation substrates and antioxidants by means of Folin-Ciocalteu reagent. Meth Enzymol, 299: 152-178.

14. Barreira, J.C.M., Ferreira, I.C.F.R., Oliveira, M.B.P.P. and Pereira, J.A. (2008). Antioxidant activities of the extract from chestnut flower, leaf, skins, and fruit. Food Chem, 107: 1106-1113.

15. Leong, L.P. and Shui, G. (2002). An investigation of antioxidant capacity of fruits in Singapore markets. Food Chem, 76: 69-75.

16. Re, R., Pelligrini, N., Proteggente, A., Pannala, A., Yong, M. and Rice-Evas, C. (1999). Antioxidant activity applying an improved ABTS radical cation decolourisation assay. Free Radic Bio Med, 26: 1231-1237.

17. Hyland, K., Voisin, E., Banoun, H. and Auclair, C. (1983). Superoxide Dismutase Assay Using Alkaline Dimethyl sulfoxide as Superoxide Anion-Generating System. Anal Biochem, 135: 280-287.

18. Bauer, AW., Kirby, W.M.M., Sherris, J.C. and Turck, M. (1966). Antibiotic susceptibility testing by a standardized single disk method. Am J Clin Pathol, 45: 493-496.

19. Mendoza MT. (1998). What's new in antimicrobial susceptibility testing? Phil J Microbiol Infect Dis, 27: 113-115.

20. Molefe-Khamanga, D.M., Mooketsi, N.A., Matsabisa, M.G. and Kensley, R.M. (2012). Qualitative phytochemical studies of solvent extracts from *Myrothamnus flabellifolius*. Online Int J Med Plant Res, 1: 1-5.

21. Dolara, P., Luceri, C., Filippo, C.D., Femi, A.P., Giovannelli, L., Caderni, G., Cecchini, C., Silvi, S., Orpianesi, C. and Cresci, A. (2005). Red wine polyphenols influence carcinogenesis, intestinal microflora, oxidative damage and gene expression profiles of colonic mucosa in F_{344} rats. Mutat Res, 591: 237-246.

22. Wink, M., Schmeller, T. and Latz-Bruning, B. (1998). Modes of action of allelochemical alkaloids: Interaction with neuroreceptors, DNA, and other molecular targets. J Chem Ecol, 24: 1881-1937.

23. Dudareva, N., Pichersky, E. and Gershenzon J. (2004). Biochemistry of plant volatiles. Plant Physiol, 135: 1893-1902.

24. Agbafor, K.N. and Nwachukwu, N. (2011). Phytochemical analysis and antioxidant property leaf extracts of *Vitex doniana* and *Mucuna pruriens*. Biochem Res Intern, 2011: 459839.

25. Amic, D., Davidovic-Amic, D., Beslo, D. and Trinajstic, N. (2003). Structure-radical scavenging activity relationship of flavonoids. Croatica Chemica Acta, 76: 55-61.

26. Alpinar, K., Ozyurek, M., Kolak, U., Guclu, K., Aras, C., Altun, M., Celik, S.E., Berker, K.I., Bektasoglu, B. and Apak, R. (2009). Antioxidant capacities of some food plants wildly grown in Ayvalik of Turkey. Food Sci Technol Res, 15: 59-64.

27. Treutter, D. (2006). Significance of flavonoids in plant resistance: a review. Environ Chem Lett, 4: 147-157.

28. Tiwari, A.K. (2001). Imbalance in antioxidant defence and human diseases: multiple approach of natural antioxidants therapy. Curr Sci, 81: 1179-1187.

29. Pallerla, P., Yellu, N.R. and Bobbala, R.K. (2019). Hepatoprotective studies on methanolic extract fractions of *Lindernia ciliata* and development of qualitative analytical

profile for the bioactive extract. Clin Phytoscience, 5: 30.

30. Imtilemla, A., Vicky, B., Samia, B.B. and Lalzikpuii, S. (2020). Preliminary phytochemical screening and in vitro antioxidant activity of the methanolic extract of *Lindernia ruellioides* (Colsm.) Pennell. Asian J Pharm Clin Res, 13: 141.

31. Wong, S.P., Leong, L.P. and William Koh, J.H. (2006). Antioxidant activities of aqueous extracts of selected plants. Food Chem, 99: 775-783.

32. Jagetia, G.C., Shetty, P.C. and Vidyasagar, M.S. (2012). Inhibition of radiation induced DNA damage by jamun, Syzygium cumini, in the cultured splenocytes of mice exposed to different doses of γ-radiation. Integr Cancer Ther, 11: 141-153.

33. Aparadh, V.T., Naik, V.V. and Karadge, B.A. (2012). Antioxidative properties (TPC, DPPH, FRAP, metal chelating ability, reducing power and TAC) within some cleome species. Ann Bot (Roma), 2: 49-56.

34. Lalrinzuali, K., Vabeiryureilai, M., Jagetia, G.C. and Lalawmpuii, P.C. (2015). Free radical scavenging and antioxidant potential of different extracts of *Oroxylum indicum in vitro*. Adv Biomed Pharm, 2: 120-130.

35. Blois, M.S. (1958). Antioxidant determinations by the use of a stable free radical. Nature, 181: 1199-1200.

36. Moon, K., Katolkar, P. and Khadabadi, S.S. (2010). In vitro antioxidant activity of methanolic extract of *Erythrinia indica*. Der Pharmacia Lettre, 2: 16-21.

37. Jagetia, G.C., Baliga, M.S., Aruna, R., Rajanikant, G.K. and Jain, V (2003). Effect of abana (a herbal preparation) on the radiation induced mortality in mice. J Ethnopharmacol, 86: 159-165.

38. Devi, N.B. and Jagetia, G.C. (2017). Free radical scavenging and antioxidant potential of different extracts of *Colocasia gigantea* (Blume) Hook. F. *In vitro*. Int Res J Pharm, 8: 72-81.

39. Miller, N.J. and Rice-Evans, C.A. (1997). Factors influencing the antioxidant activity determined by the ABTS+ radical cation assay. Free Radic Res, 26: 195-199.

40. Re, R., Pellegrini, N., Proteggente, A., Pannala, A. and Yang, M. (1999). Antioxidant activity applying an improved ABTS radical cation decolorization assay. Free Rad Biol Med, 26: 1231-1237.

41. Park, E.J. and Pezzutto, J.M. (2002). Botanicals in cancer chemoprevention. Cancer Metastasis Rev, 21: 231-255.

42. Yen, G.C. and Duth, P.D. (1994). Scavenging effect of methanolic extracts of peanut hulls on free radical and active-oxygen species. J Agric Food Chem, 42: 629-632.

43. Robak, J. and Gryglewski, R.J. (1998). Flavonoids are scavengers of superoxide anions. Biochem Pharmacol, 37: 837-841.

44. Hassine, D.B., Abderrabba, M., Yvon, Y., Lebrihi, A., Mathieu, F., Couderc, F. and Bouajila, J. (2012). Chemical Composition and *in-vitro* evaluation of the antioxidant and antimicrobial activities of *Eucalyptus gilli* essential oil and extracts. Molecules, 17: 9540-9558.

45. Okwu, D.E. and Okwu, M.E. (2004). Chemical composition of *Spondias mombin* Linn. Plant Parts. J Sustain Agric Environ, 6: 140-147.

46. Qader, M.K., Khalid, N.S. and Abdullah, A.M. (2013). Antibacterial activity of some plant extracts against clinical pathogens.

Int J Microbiol Immunol Res, 1: 53-56.

47. Verma, V., Singh, R., Tiwari, R.K., Srivastava, N. and Verma, A. (2012). Antibacterial activity of extracts of Citrus, Allium & Punica against food borne spoilage. Asian J Plant Sci Res, 2: 503-509.

48. Mahboubi, A., Asparpanah, J., Sadaghiqani, P.N. and Faizi, M. (2015). Total phenolic and flavonoid content and antibacterial activity of *Punica granatum* L. Var. pleniflora flower (golnar) against bacterial strains causing food borne diseases. BMC Complem Altern Med, 15: 366-373.

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LC- HRMS and Phytochemicals Analysis of *Gnidia glauca* L. leaf crude extract with different solvents

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Abstract

The member of the Thymelaeaceae family, *Gnidia glauca* (Fresen) Gilg, has been found to possess a multitude of traditional phytomedicinal and agrochemical applications. When viewed in its entirety, *Gnidia glauca* L. (Thymelaeaceae) stands as the most extensive genus within the family, boasting a remarkable 140 to 160 species scattered across Africa, Arabia, India, Madagascar, and Sri Lanka. The leaves of *Gnidia glauca* L., which are meticulously extracted using chloroform, methanol, and alcohol, yield a diverse array of chemically intricate and biologically potent substances. A staggering number of over 90 compounds have been successfully identified within it. The meticulous phytochemical analysis of *Gnidia glauca* leaves has revealed the presence of alkaloids, phenols, flavonoids, tannins, glycosides, saponins, terpenoids, and steroids. The objective of this endeavor is to employ LC-HRMS-based techniques to meticulously analyze and detect both targeted and non-targeted phenolic compounds derived from the leaves of *Gnidia glauca* L.

Keywords: *Gnidia glauca* L, Thymelaeaceae, extraction, phytochemical profile, LC-HRMS.

Introduction

Gnidia glauca L. is an indigenous botanical specimen possessing a rather intricate chemical composition encompassing alkaloids, flavonoids, saponins, steroids, organic acids, polysaccharides, and diverse trace elements. These compounds exhibit advantageous properties such as anti-inflammatory effects, pain alleviation, inhibition of tumor cell proliferation, and more. Owing to the deleterious side effects and health hazards associated with chemically synthesized medications, the emergence of complementary and alternative medicine has transpired. Medicinal plants have garnered significant attention due to their biocompatibility and efficacy. *Gnidia glauca* L. holds a prominent position in the realm of mythology, traditional phytomedicine, and agrochemical applications worldwide.

In addition to its utilization in the treatment of tumors, wounds, snake bites, sore throats, and burns, *Gnidia glauca* L. is renowned for its piscicidal, insecticidal, molluscidal, and even homicidal properties when employed as arrow poisons. Furthermore, its antineoplastic activity is purportedly remarkably superior (17). Phytochemical investigations of *Gnidia glauca* L. have unveiled the presence of toxic diterpene esters of the daphnane variety, which represent the principal orthoesters found in plants and exhibit remarkable biological activities, including antineoplastic and cytotoxic effects. *Gnidia glauca* L. is abundant in diterpene esters, coumarins, flavonoids, chromones, lignans, and neolignans (3). These chemical

constituents of *Gnidia glauca* L. hold potential as foundational materials for the synthesis of analogues capable of treating a diverse array of ailments.

Materials and Methods

Collection and identification of plant

Gnidia glauca (Fresen) Gilg was collected from Nivkane village of Patan, Maharashtra. The plant was authenticated from Dept. of Botany, Shivaji University Kolhapur and voucher specimens (VSN/ 001) were deposited in the department for future reference.

Extraction of plant material using solvents

The leaves of *Gnidia glauca* (Fresen) Gilg were thoroughly cleansed on four to five occasions with flowing water from the faucet and once with sterile water prior to being dried in the shade, pulverized, and utilized for extraction. A quantity of 20 grams of powdered plant material underwent a rigorous 48-hour (1:10) soxhlet extraction employing the solvents methanol, chloroform, and alcohol. Subsequently, the extracts were filtered through Whatman filter paper No. 1 and dried in a water bath shaker. In order to ascertain the presence of various secondary metabolites, all of the extracts were subjected to preliminary phytochemical screening and LC-HRMS analysis (24).

Preliminary analysis of *Gnidia glauca L.* leaf extracts

The concentrates were subjectively tested and assessed for the existence of various phytochemical constituents employing standard methodologies to identify the constituents as delineated by Harborne (9).

Test for alkaloids

Dragondroff's test **–** Crude extract was mixed with Dragondroff's reagent (potassium bismuth iodide solution). Reddish brown precipitate was formed which suggested the presence of alkaloids. 5 ml of the extract was added to 2 ml of HCl. To this acidic medium, 1 ml of Wagner's reagent was added. A reddish precipitate brown produced immediately indicates the presence of alkaloids.

Test for flavonoids

1.5 ml of a 50% methanol solution was used to treat 4 ml of the extract solution. Metal magnesium was added after the solution was warmed up. 5 to 6 drops of concentrated hydrochloric acid were added to this solution, and flavonoids with a red color and flavones with an orange color were observed. 2ml of extract solution add with 2 ml of diluted 10% sodium hydroxide (NaOH), golden yellow precipitate shows presences of flavonoids.

Test for glycosides

1 ml of Fehling's solution was heated and added to a small amount of extract. An orange precipitate indicates the presence of glycosides.

Test for tannins

Ferric chloride reagent added to the filtrate. The presence of tannins was confirmed by a precipitate that was green, blue-green, or blue-black. 2 to 3ml of extract solution and add 10% ferric chloride reagent green, blue-green, or blue-black precipitate occur it shows presences of tannins

Test for saponins

The saponins were found using the frothing test. In a water-filled test tube, 0.5 grams of extract from each part was taken. The presence of saponins is indicated by the mass of bubbles produced when the solutions were warmed up. Foam test – 2ml of extract solution and add 20 ml of distilled water and shake vigorously wait for 3 min formation of honeycomb like froth shows the presences of saponins.

Test for quinones

To a modest quantity of extract solution, sulphuric acid is added. Quinones are identified

by their red appearance.

Test for terpenoids

2 ml of chloroform was mixed with 5 ml of each extract. To make a layer, 3 ml of concentrated H_2SO_4 was added. Terpenoids were detected by the formation of a reddish-brown precipitate at the interface.

Test for phlobatannins

When each plant sample's aqueous extract was boiled in 1% aqueous hydrochloric acid, a red precipitate fell as evidence for the Phlobatannins.

Test for steroid

Chloroform and 2.5 ml of acetic anhydride were used to treat the extract. After that, a concentrated solution of sulphuric acid was slowly added, and the terpenoids' reddish-violet color and the steroids' greenish-blue color were observed. Then 2 ml extract solution dissolved in 2 ml of chloroform and add concentrated H_2SO_4 side by side carefully red precipitate shows that steroid is present. 2 ml of extract solution then add 1 ml of ethyl acetate and mix into 2 ml of chloroform and few drops of concentrated H_2SO_4 side by side carefully red precipitate shows that steroid is present.

Liquid chromatography high resolution mass spectrometry (LC-HRMS) characterization

The determination of bioactive compounds in the leaf extracts was conducted at the esteemed Center for Applications in Mass Spectrometry (CAMS), Venture Laboratory, located in Pune. This analysis was performed utilizing the sophisticated LC-HR-MS technique (Liquid Chromatography-High Resolution-Mass Spectrometry). Prior to analysis, the methanolic, chloroformic, and alcoholic extracts underwent centrifugation at a speed of 12,000 revolutions per minute for duration of 10 minutes. The HPLC system employed consisted of two pumps and an automated injector. The separation process was accomplished using an Agilent Q-ToF G6540B instrument, which was connected to an Agilent 1260 Infinity II HPLC system equipped with an Agilent Eclipse XDB-C18 column measuring 3X150 mm and possessing a particle size of 3.5 microns. Two distinct mobile phases were employed: A-0.1% formic acid in water and B-0.1% formic acid in acetonitrile, with a flow rate of 0.3 mL/min. The LC conditions were as follows: an initial concentration of 5% in phase B from 0 to 3 minutes, followed by a linear increase from 5% to 95% between 2 and 25 minutes, maintaining 5% to 95% from 25 to 28 minutes, and transitioning from 5% to 95% between 25 and 28.1 minutes. Finally, the concentration returned to 0% to 5% during the interval of 28.1 to 30 minutes. For the MS analysis, the Dual AJS ESI Mass spectrometer was employed in positive ionization mode, utilizing data-dependent automatic switching between MS and MS/MS acquisition modes.

Results and Discussion

Table No. 1.Qualitative phytochemical analysis of *Gnidia glauca* leaf in various solvents.

Sr. No	Metabolites	*Gnidia glauca* leaves		
		Methanol	Alcohol	Chloroform
1	Alkaloid	+ve	+ve	+ve
2	Flavonoids	+ve	+ve	+ve
3	Glycosides	-ve	+ve	-ve
4	Tannins	+ve	+ve	-ve

5	Saponins	+ve	+ve	+ve
6	Quinones	+ve	+ve	-ve
7	Terpenoids	+ve	-ve	+ve
8	Phlobatannins	-ve	-ve	-ve
9	Steroid	+ve	+ve	+ve

Note – (+ve) present, (-ve) absent

Table 2. Phytochemicals screening of solvent (Alcohol) extracts of *Gnidia glauca* leaves by LC-HRMS

Sr. No	Compound Name	Regulator / Inhibitor	Chemical formula	Rt. Time	Mass	Score	Importance
1	3-Phenylpropyl glucosinolate	Inhibitor	$C_{16}H_{23}NO_9S_2$	2.356	437.0802	91.60	Antimicrobial activity, in vivo cytotoxicity
2	Zwittermicin A	Inhibitor	$C_{13}H_{28}N_6O_8$	3.805	396.1969	97.46	Suppress plant disease
3	Famciclovir	Inhibitor	$C_{14}H_{19}N_5O_4$	3.902	321.1427	93.53	Herpes virus
4	Dimepiperate	Inhibitor	$C_{15}H_{21}NOS$	3.973	263.1353	90.68	Moderately hazardous class II (Herbicide)
5	Pyranodelphinin A	Inhibitor	$C_{30}H_{33}O_{16}$	7.388	649.1774	98.12	Natural fungicide
6	N-heptanoyl-homoserine lactone	Regulator/ Inhibitor	$C_{11}H_{19}NO_3$	10.327	213.1355	94.18	Regulation of virulence, infection prevention, and septicemia in fish.
7	Erinapyrone C	Inhibitor/ regulator	$C_8H_{10}O_5$	12.215	186.0537	92.41	Cytotoxicity of HeLa cells and nerve growth
8	Kiwiionoside	Inhibitor	$C_{19}H_{34}O_9$	12.393	406.2202	97.00	Antirepellent compound
10	(+)-Gallocatechin	Inhibitor	$C_{15}H_{14}O_7$	14.061	306.0742	98.96	Insecticidal properties
11	Ginkgolide C	Inhibitor	$C_{20}H_{24}O_{11}$	14.333	440.1316	99.67	Pesticidal properties
12	Garcimangosone D	Inhibitor	$C_{19}H_{20}O_9$	14.701	392.1105	98.19	Pesticidal properties
13	Khellolglucoside	Inhibitor	$C_{19}H_{20}O_{10}$	16.390	408.1058	92.61	Pesticidal properties
14	6-Undecanone	Inhibitor	$C_{12}H_{22}O$	16.508	170.1678	96.28	Pesticidal properties

15	Lansiuma-mide C	Inhibitor	$C_{18}H_{19}NO$	17.076	265.1471	96.32	Pesticidal properties
16	3R-hy-droxy-oct-adecanoic acid	Inhibitor	$C_{18}H_{36}O_3$	25.019	300.2663	98.63	Antioxidant, hypo-cholesterolemic, nematicide, pesticide, 31antiandrogenic, flavour, hemolytic, 5-alpha reductase inhibitor
17	10-Hydroxy-myristic acid methyl	Inhibitor	$C_{15}H_{30}O_3$	24.558	258.2196	95.6	Antioxidant, hyper-cholesterolemic,-cancer-preventive, cosmetic, nematicid.
18	Cyclic de-hy-poxanthine futalosine	Inhibitor	$C_{14}H_{14}O_7$	17.499	294.0744	98.18	Enzymatic proper-ties. Efficient growth of the *Streptomyces coelicolor* mutant

Table No. 3. Phytochemicals screening of solvent (Methanol) extracts of *Gnidia glauca* leaves by LC-HRMS

Sr. No	Compound Name	Regulator / Inhibitor	Chemical formula	Rt. Time	Mass	Score	Importance
1	N'-Hydroxyneosaxi-toxin	Inhibitor	$C_{10}H_{17}N_7O_6$	3.392	331.1248	92.52	Paralysis the nerve cells
2	Mycinamicin IV	Inhibitor	$C_{37}H_{61}NO_{11}$	15.932	695.4258	90.43	Anticancer
3	Lycoperoside D	Inhibitor	$C_{39}H_{65}NO_{12}$	16.182	739.4513	94.53	Inflammatory diseases, Skin health
4	Aloesol 7-glucoside	Inhibitor	$C_{19}H_{24}O_9$	17.265	396.1415	93.68	Inflammation and ulcer
5	Tiamulin	Inhibitor	$C_{28}H_{47}NO_4S$	20.119	493.3241	92.50	Antibacterial drug
6	3-Feruloyl-1,5- quino-lactone	Inhibitor	$C_{17}H_{18}O_8$	20.071	350.1001	98.48	Biomass deg-radation
7	4,4-Difluoro-pregn-5-ene-3,20- di-one	Inhibitor	$C_{21}H_{28}F_2O_2$	20.889	350.2046	93.53	induced aller-gic rhetis
8	Nicotine imine	Regulator	$C_{10}H_{13}N_2$	28.037	161.1081	92.98	Biomarker for oral cancer pa-tient
9	16-hydroxy hexadeca-noic acid	Regulator	$C_{16}H_{32}O_3$	24.538	272.2360	93.07	steriodal estro-gen

10	Prasterone sulfate	Inhibitor	$C_{19}H_{28}O_5S$	3.260	368.1658	96.10	It is a universal precursor for the peripheral local production and action of estrogens and androgens in target tissues such as brain, bone, skin, and adipose tissue.
11	Zwittermicin A	Inhibitor	$C_{13}H_{28}N_6O_8$	3.805	396.1969	97.46	Suppress plant disease
12	Famciclovir	Inhibitor	$C_{14}H_{19}N_5O_4$	3.902	321.1427	93.53	Herpes virus
13	Decarbamoylneosaxitoxin	Regulator	$C_9H_{16}N_6O_4$	9.866	272.1244	93.73	Neurotoxin
14	Musca-aurin-VII	Regulator	$C_{15}H_{17}N_4O_6$	10.989	349.1141	97.25	contribute to the pigment pattern of fly agarics
15	Hydralazine	Inhibitor	$C_8H_8N_4$	11.258	160.0750	98.18	Therapy for hypertension
16	Kiwiionoside	Inhibitor	$C_{19}H_{34}O_9$	12.393	406.2202	97.00	Anti-repellent compound
17	(+)-Gallocatechin	Inhibitor	$C_{15}H_{14}O_7$	14.061	306.0742	98.96	Insecticidal
18	Ginkgolide C	Inhibitor	$C_{20}H_{24}O_{11}$	14.333	440.1316	99.67	Pesticidal
19	Garcimangosone D	Inhibitor	$C_{19}H_{20}O_9$	14.701	392.1105	98.19	Pesticidal
20	Khellolglucoside	Inhibitor	$C_{19}H_{20}O_{10}$	16.390	408.1058	92.61	Pesticidal
21	10,16-dihydroxy-palmitic acid	Inhibitor	$C_{16}H_{32}O_4$	21.932	288.2301	96.10	Antioxidant, hypocholesterolemic, nematicide, pesticide, antiandrogenic, flavor, hemolytic, 5-alpha reductase inhibitor
22	Cyclic de-hypoxanthine futalosine	Inhibitor	$C_{14}H_{14}O_7$	17.499	294.0744	98.18	Enzymatic properties. Efficient growth of the *Streptomyces coelicolor* mutant

LC- HRMS and Phytochemicals analysis of *Gnidia glauca* L. leaf crude extract with different solvents

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Table No.4 Phytochemicals screening of solvent (Chloroform) extracts of *Gnidia glauca* leaves by LC-HRMS

Sr. No	Compound Name	Regulator / Inhibitor	Chemical formula	Rt. Time	Mass	Score	Importance
1	cortisol 21-sulfate	Regulator	$C_{21}H_{30}O_8S$	3.137	442.1657	97.17	Affects behavior through its direct action on the central nervous system, its effects on intermediary metabolism or negative feedback on pituitary ACfH release.
2	ACRL Toxin II	Inhibitor	$C_{17}H_{24}O_5$	17.328	308.1629	96.05	host specific pathotoxic, plant and animal cell death
3	Mycinamicin IV	Inhibitor	$C_{37}H_{61}NO_{11}$	15.932	695.4258	90.43	Anticancer
4	Pitheduloside A	Inhibitor	$C_{41}H_{66}O_{13}$	16.434	766.4512	94.91	Larvicidal and ovicidal activity
5	Tiamulin	Inhibitor	$C_{28}H_{47}NO_4S$	20.119	493.3241	92.50	Antibacterial drug
6	Nicotine imine	Regulator	$C_{10}H_{13}N_2$	28.037	161.1081	92.98	Biomarker for oral cancer patient
7	16-hydroxy hexadecanoic acid	Regulator	$C_{16}H_{32}O_3$	24.538	272.2360	93.07	steriodal estrogen
8	3 Irinotecan	Inhibitor	$C_{33}H_{38}N_4O_6$	28.415	586.2772	93.40	Cancer treatment
9	Famciclovir	Inhibitor	$C_{14}H_{19}N_5O_4$	3.902	321.1427	93.53	Herpes virus
10	(+)-Gallocatechin	Inhibitor	$C_{15}H_{14}O_7$	14.061	306.0742	98.96	Insecticidal
11	Garcimangosone D	Inhibitor	$C_{19}H_{20}O_9$	14.701	392.1105	98.19	Pesticidal
12	3R-hydroxy-octadecanoic acid	Inhibitor	$C_{18}H_{36}O_3$	25.019	300.2663	98.63	Antioxidant, hypocholesterolemic, nematicide, pesticide, antiandrogenic, flavour, hemolytic, 5-alpha reductase inhibitor
13	3-Hydroxyindolin-2-one	Inhibitor	$C_8H_7NO_2$	5.161	149.0474	98.00	Allergenic, anesthetic, antibacterial, anticancer, antimutagenic, antipeptic, antiseptic, antispasmodic, antitumor, candidicide, flavour, insecticide, nematicide, pesticide, sedative, termiticide, tyrosinase inhibitor
14	1-(m - Methoxycinnamoyl) pyrrolidine	Inhibitor	$C_{14}H_{17}NO_2$	13.110	231.1249	94.64	Allergenic, anesthetic, antiodontalgic, antipruritic, antiseptic, flavour, fungicide, pesticide, sedative.

15	Cyclic de-hypox-anthine futalosine	Inhibitor	$C_{14}H_{14}O_7$	17.499	294.0744	98.18	Enzymatic properties. Efficient growth of the *Streptomyces coelicolor* mutant
16	Proglumide	Inhibitor	$C_{18}H_{26}N_2O_4$	16.767	334.1885	91.98	Inhibited gastrin-stimulated growth. (Treatment of stomach ulcers)

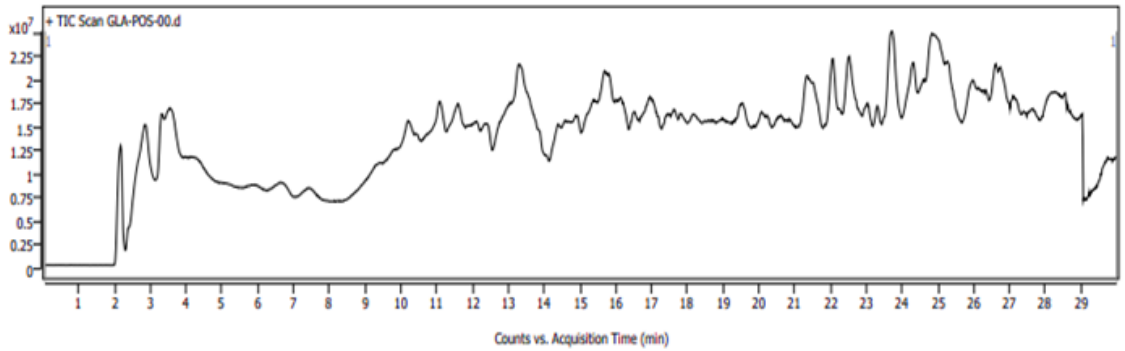

Fig. 1. LC-HRMS of *Gnidia glauca* Leaves Alcohol extract

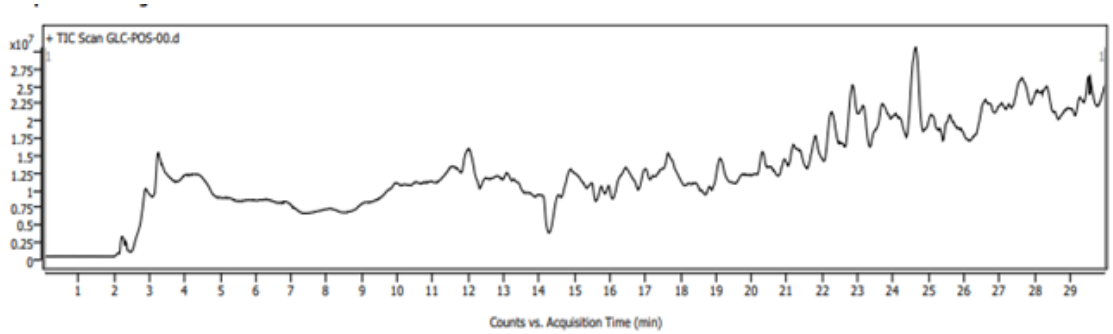

Fig. 2. LC-HRMS of *Gnidia glauca* Leaves Chloroform extract

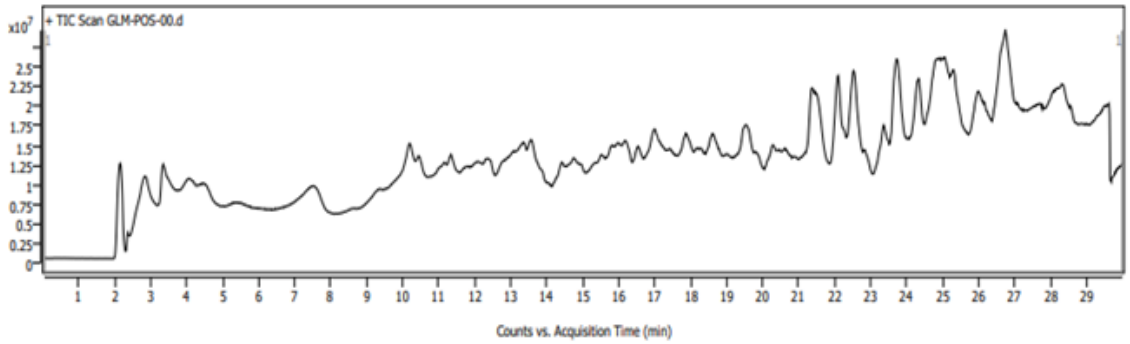

Fig. 3. LC-HRMS of *Gnidia glauca* Leaves Methanol extract

LC- HRMS and Phytochemicals analysis of *Gnidia glauca* L. leaf crude extract with different solvents

Discussion

A plethora of phytochemical screenings of botanical specimens has been conducted, unveiling the presence of myriad clusters of chemical compounds (1). It has also been documented that rotenone, a chemical constituent discovered within *Gnidia glauca*, possesses ichthyotoxic properties and possesses the remarkable ability to induce the excruciating loss of teeth. It appears that various phytochemicals present in *Gnidia glauca* are exerting an influence, as evidenced by numerous scholars. *Gnidia glauca* hampers the activity of amylase and glycosidase, yielding favorable outcomes (6).

Components of phytochemistry

Investigation of phytochemicals was conducted in three solvents of the leaf of *Gnidia glauca* (Table no. 1). When compared to the extracts of *Gnidia glauca* prepared with alcohol, chloroform, or methanol, there exist numerous medical applications for these compounds. The range of secondary metabolites produced by *Gnidia glauca* is delineated below, alongside some of their biological functions. Due to their extensive employment in medicine, *Thymelaeaceae* plants have been the subject of phytochemical studies, and there have also been reports on their toxicity (5).

The phytochemicals in three preparations of *Gnidia glauca* leaves were qualitatively analyzed. The methanolic extract of *Gnidia glauca* exhibited the highest extraction of phytochemicals when compared to chloroform and alcohol extracts. The phytochemical screening of all leaf extracts unveiled the presence of flavonoids, steroids, tannins, and terpenoids. The phytochemical screening reveals that these chemicals possess the potential to be employed for a variety of therapeutic purposes.

Methanol, chloroform, and alcohol were utilized to extract 20 grams of powdered dry leaves, with 200 milliliters of solvents. The leaves of *Gnidia glauca* yielded a total of 1-2 grams of crude extract. One gram of the crude extract was utilized for solvent partition using solvents with varying polarities. In this investigation, solvents with increasing polarity such as chloroform, alcohol, and methanol were employed. The solvent partition was repeated until the solvent in the thimble became clear, indicating the completion of the extraction process. When compared to the other partitions, the methanol partition yielded the highest amount. This finding revealed the presence of polar chemicals in *Gnidia glauca* leaves. LC-HRMS analysis was performed on each partition. LC-HRMS analysis was employed to identify the chemical components found in *Gnidia glauca* leaf extracts. Table 2 enumerates the active principles in solvent extracts of *Gnidia glauca* leaves, alongside their retention time (RT), molecular formula, and molecular mass (MW).

Phytochemical profiling using LC-HRMS

The foliage of *Gnidia glauca* was individually extracted utilizing the Sohxlet extraction technique with three dissimilar polarity solvents (chloroform, methanol, and alcohol). The LC-HRMS data (retention time, molecular formula, and m/z) of the tentatively annotated peaks are documented in Tables 2, 3, and 4 subsequent to the generation of the extracts being subjected to comprehensive LC-HRMS investigations. On-line databases (METLIN, KNApSacK, PubChem, NIST Chemistry WebBook) or earlier literature reporting on the LC-MS analysis of phytochemicals were employed to compare the present study. All of these compounds were discovered in the alcoholic extract of *Gnidia glauca* leaves, namely 3-Phenylpropyl glucosinolate, DL-2-Aminoadipic acid, Prasterone sulfate, N'-Hydroxyneosaxitoxin, Zwittermicin A, Famciclovir, Dimepiperate, Monoglyceride citrate, Pyranodelphinin A, N2,N2-Dimethylguanosine, Decarbamoylneosaxitoxin, (R) Pantothenic acid 4'-O-b-D-glucoside, N-heptanoyl-homoserine lactone, Musca-aurin-VII, Trichotomine, Hydralazine, Erinapyrone C, Kiwiionoside, (+)-Gallocatechin, Ginkgolide, Garcimangosone D, Khellolglucoside, 6-Un-

decanone, Lansiumamide C, Aloesol 7-glucoside, 3-Feruloyl-1,5-quinolactone, Nicotine imine, 16-hydroxy hexadecanoic acid, 3R-hydroxy-octadecanoic acid, 10-Hydroxymyristic acid methyl, Cyclic de-hypoxanthine futalosine. In the methanolic extract, N'-Hydroxyneosaxitoxin, Mycinamicin IV, Lycoperoside D, Aloesol 7-glucoside, Tiamulin, 3-Feruloyl-1,5-quinolactone, 4,4-Difluoropregn-5-ene-3,20-dione, Nicotine imine, 16-hydroxy hexadecanoic acid, Prasterone sulfate, Zwittermicin A, Famciclovir, Decarbamoylneosaxitoxin, Musca-aurin-VII, Hydralazine, Kiwiionoside, (+)-Gallocatechin, Ginkgolide C, Garcimangosone D, Khellol-glucoside, 10,16-dihydroxy-palmitic acid, Cyclic de-hypoxanthine futalosine were present. Meanwhile, in the chloroformic extract, the compounds identified were cortisol 21-sulfate, ACRL Toxin II, Mycinamicin IV, Pithedu-loside A, Tiamulin, Nicotine imine, 16-hydroxy hexadecanoic acid, 3 Irinotecan, Famciclovir, (+)-Gallocatechin, Garcimangosone D, 3R-hydroxy-octadecanoic acid, 3-Hydroxyindo-lin-2-one, 1-(m-Methoxycinnamoyl) pyrrolidine, Cyclic de-hypoxanthine futalosine, Proglumide.

Some of the compounds were present in all three extracts and exhibited similar activity. Recently, *Gnidia glauca* leaves have been documented. The overall phenolic content of the active extracts (126.25 0.20 g GAE/mg) and flavonoid content (25.75 0.10 g CE/mg) were both substantial (25). Figure 1, Figure 2, and Figure 3 illustrate the peak of the compound.

Antioxidant properties

The primary phenolic content of the methanolic extract derived from the leaf *of Gnidia glauca*, which possesses noteworthy antioxidant properties, amounted to 203.3 GAE/g. Its IC50 for ABTS was 16.3 g/mL, while for nitric oxide radical scavenging it was 360.8 g/mL. Furthermore, at the 30-minute mark, a FRAP value of 993.7 m TE/mg was observed, and the total antioxidant activity was evaluated at 142.5 mg AAE/g (27). Similar patterns were observed in the alcoholic extracts of *Gnidia glauca* leaf, which exhibited substantial phenolic and flavonoid content. The alcoholic extracts of *Gnidia glauca* leaves demonstrated second order rate constants of 3.73 106 in the case of pulse radiolysis-generated hydroxyl radical scavenging. The leaf extract in methanol displayed effective DPPH, superoxide, and nitric oxide radical scavenging activities (7).

Pesticidal and larvicidal properties

In Kenya, the utilization of *Gnidia glauca* leaves as an insecticide has been documented (13). The eggs of the teak defoliator *Hyblaea puera* Cramer were significantly eradicated by an aqueous extract derived from the leaves and bark of *Gnidia glauca*, with mortality rates reaching 44.4% and 45.7%, respectively (20). To explore the antileukemic and piscicidal properties of *Gnidia glauca*, dried ground roots were subjected to extraction using 95% ethanol at ambient temperature, while being gently agitated for duration of 24 hours. In order to isolate the piscitoxic fraction, known as gnidiglaucin ($C_{32}H_{46}O_{10}$), the extract was subsequently partitioned using varying proportions of a chloroform-water mixture. However, the isolated compound did not exhibit any inhibitory effects in an in-vivo test for antileukemic activity (P388) (34). Hexane and chloroform extracts obtained from the dried bark of *Gnidia glauca* demonstrated a modest larvicidal effect on mosquito larvae, whereas extracts derived from the fresh bark of the plant exhibited more pronounced larvicidal effects on *Aedes aegypti* second instar larvae. Remarkably, the chloroform extract of fresh bark displayed its maximum activity, resulting in 100% mortality, within a matter of minutes. Bioassay-guided fractionation revealed that the larvicidal activity is primarily attributed to substances such as bicoumarin and Pimelea factor P2 (2). The methanolic extract of *Gnidia glauca* leaves exhibits properties that are beneficial in the management of diabetes (6). Among the leaf extracts of *Vitex negundo*, the 1% chloroform extract demonstrates the highest larval mortality rate (10.30%), surpassing the acetone leaf extract

(7.7%) and the methanol leaf extract (3.09%) (32). Furthermore, it outperforms the artificial diet, which is both costly and time-consuming, in investigating growth factors necessary for the development of early-stage larvae (21).

Antimicrobial properties

Plant pathogenic fungi are the main reason for significant losses in crop output as well as in the farmers' income. Thus, it is crucial to produce low-cost, herbal antifungal medications that are also environmentally friendly. A plant pathogenic fungus called *Phytophthora parasitica*, which causes pineapple heart rot, was variably inhibited by aqueous extracts of different sections of *Gnidia glauca*. The *Gnidia glauca* seeds, leaves, and barks displayed an inhibition up to 19.16, 15.90, and 23.46%, respectively, at a concentration of 5%. Similar to this, increased activity was seen at 10%, which is equivalent to 28.47, 34.59, and 33.60% for seed, leaves, and bark, respectively (20). Recently, a strong anticariogenic activity of the methanolic extract of *Gnidia glauca* leaves against *Streptococcus mutans* was discovered. The total phenolic and flavonoid contents of the active extracts were both high (126.25 0.20 g GAE/ mg and 25.75 0.10 g CE/mg, respectively) (12). When compared to leaf and flower extracts, *Gnidia glauca* bark extract has greater antibacterial action against pathogens that cause urinary tract infections, such as *Escherichia coli, Klebsiella pneumoniae, Pseudomonas aeruginosa, Staphylococcus aureus,* and *Enterococcus faecalis* (25).

Nano particles properties

The flower extract of *Gnidia glauca* facilitates the production of gold nanoparticles owing to its abundant phenolic and flavonoid constituents. This process exhibits remarkable expeditiousness, as it can be accomplished entirely within a time frame of fewer than 20 minutes. The ultimate form of the AuNPs consists of diminutive spheres with a median diameter of 10 nm. Through the utilization of high resolution transmission electron microscopy and various other methods of characterization, peculiar structures such as Nano-triangles were also observed (6).

Conclusion

Gnidia glauca, an eminent botanical specimen, possesses remarkable ethno medicinal properties, encompassing anticoagulant, antioxidant, and anthelmintic activities. Furthermore, it serves as a biomarker for oral cancer patients undergoing cancer treatment. This multifaceted plant exhibits allergenic, anesthetic, antibacterial, anticancer, ant mutagenic, antiseptic, antispasmodic, antitumor, and tyrosinase inhibitory qualities. Additionally, it functions as a flavor enhancer, insecticide, nematicide, pesticide, sedative, and termiticide, while also possessing antipruritic and fungicidal attributes. Notably, it demonstrates host-specific pathotoxicity, leading to cellular demise in both plant and animal organisms. Moreover, it showcases antioxidant and hypocholesterolemic properties, alongside its nematicidal, pesticidal, and antiandrogenic effects. It imparts a delightful flavor and exhibits hemolytic and 5-alpha reductase inhibitory activities. Furthermore, it displays commendable insecticidal, herbicidal, and fungicidal properties, as well as mild antifungal activity. Phytochemical investigations have unequivocally established the indispensability of *Gnidia glauca* in the daily lives of humans, animals, and plants, ensuring their well-being and vitality.

Conflict of interest

The authors have no conflicts of interest regarding this investigation**.**

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References

1. Ajayi, I. A., Ajibade, O., & Oderinde, R. A.

(2011). Preliminary phytochemical analysis of some plant seeds. *Res J ChemSci*, *1*(3), 58-62.

2. Amarajeewa, B. W. R. C., Mudalige, A. P., & Kumar, V. (2007). Chemistry and mosquito larvicidal activity of *Gnidia glauca*. *PERADENIYA UNIVERSITY RESEARCH SESSIONS PURSE 2007 Volume 12 Part I-Agricultural, Biological and Medical Sciences Editorial Board*, 101.

3. Bhandurge, P., Rajarajeshwari, N., Ganapaty, S., &Pattanshetti, S. (2013). The Gnidia genus: a review. *Asian Journal of Biomedical and Pharmaceutical Sciences*, *3*(19), 1.

4. Bjorklund J, Leete E. Biosynthesis of the benzoyl moiety of cocaine from cinnamic acid via (R)-(+)-3-hydroxy-3- phenylpropanoic acid. Phytochemistry 1992; 3:357–60.

5. Borris, R. P., Blaskó, G., & Cordell, G. A. (1988). Ethnopharmacologic and phytochemical studies of the Thymelaeaceae. *Journal of ethnopharmacology*, *24*(1), 41-91.

6. Ghosh, S., Ahire, M., Patil, S., Jabgunde, A., BhatDusane, M., Joshi, B. N., &Chopade, B. A. (2012). Antidiabetic activity of Gnidia glauca and Dioscoreabulbifera: potent amylase and glucosidase inhibitors. *Evidence-Based Complementary and Alternative Medicine*, *2012*.

7. Ghosh, S., Derle, A., Ahire, M., More, P., Jagtap, S., Phadatare, S. D., &Chopade, B. A. (2013). Phytochemical analysis and free radical scavenging activity of medicinal plants Gnidia glauca and Dioscoreabulbifera. *PLoS One*, *8*(12).

8. Ghosh, S., Patil, S., Ahire, M., Kitture, R., Gurav, D. D., Jabgunde, A. M., ...&Chopade, B. A. (2012). Gnidia glauca flower extract mediated synthesis of gold nanoparticles and evaluation of its chemocatalytic potential. *Journal of Nanobiotechnology*, *10*, 1-9.

9. Harborne, A. J. (1998). *Phytochemical methods a guide to modern techniques of plant analysis*. Springer science & business media.

10. Higashi, O., Takagaki, T., Koshimizu, K., Watanabe, K., Kaji, M., Hoshino, J., ...&Muanza, D. N. (1991). Biological activities of plant extracts from tropical Africa. *African Study Monographs*, *12*(4), 201-210.

11. Huges TA, Heimberg M, Wang X. Comparative lipoprotein metabolism of myrisate-palmitate and stearate in normalipidemic man metabolism. Int J Res Pharm Sci 1996;5(9):1108-18.

12. Junaid S, Dileep N, Rakesh KN, Pavithra GM, Vinayaka KS, et al. (2013) Anticariogenic activity of Gnidia glauca (Fresen.) Gilg, Pothosscandens L. and Elaegnuskologa Schlecht. J App Pharm Sci 3: 020-023.

13. Kareru, P. G., Kenji, G. M., Gachanja, A. N., Keriko, J. M., &Mungai, G. (2007). Traditional medicines among the Embu and Mbeere people of Kenya. *African Journal of Traditional, Complementary and Alternative Medicines*, *4*(1), 75-86.

14. Khan A, Usman R, Wang M, Rauf A, Muhammad N, Aman A, Tahir TH. In vitro biological screening of the stem of Desmodiumelegans. Asian Pacific J Tropical Biomedicine 2013;3(9):711-5.

15. Kharat, S. S., Kumkar, P. B., Rajpure, S. R., &Sonawane, K. S. (2013). Qualitative phytochemical screening of Gnidia glauca (Fresen) Gilg. plant extract. *International Journal of Pharma and Bio Sciences*, *4*(4).

16. Lucie AT, Dogo S, Lakouetene DB, Florent BO, Talla GM, Anna T, et al. Chemical characterization and insecticidal activity of ethyl acetate and dichloromethane extracts of Drypetesgossweileri against Sitophilus zeamais, Triboliumcastaneum and Rhyzoperthadominica. J Life Sci 2013;7(10):1030-40.

17. Mabberley, D. J. (2017). *Mabberley's plant-book: a portable dictionary of plants, their classification and uses* (No. Ed. 4). Cambridge university press.

18. Mingarro I, Lukovic D, Vilar M, Perez-Gil J. Synthetic pulmonary surfactant preparations: new developments and future trends. Current Medicinal Chemistry 2008;15(4):393-403.

19. Mulyono N, Bibiana WL, Ocktreya L, Rahayu S. Antidiarrheal activity of apus bamboo (Gigantochloaapus) leaf extract and its bioactive compounds. American J Microbiol 2013;4(1):1-8.

20. Naik, L. K. (2007). Ovicidal properties of plant extracts against the eggs of teak defoliator, Hyblaeapuera Cramer. *Karnataka Journal of Agricultural Sciences*, *20*(2), 291-293.

21. Netam, V., & Shewale, V. (2022). Study of Biology of Leucinodes orbonalis Guenee (EFSB) Under Laboratory Condition Fed on Two Different Diets. *ZeichenJournal* , 8 (6), 834-837.

22. Nethravathi, H. R., Kekuda, T. P., Vinayaka, K. S., Thippeswamy, N. B., Sudharshan, S. J., & Kumar, S. V. P. (2010). Studies on antioxidant and anthelmintic activity of Gnidia glauca (Fresen) Gilg. *Asian Journal of Bio Science*, *5*(1), 6-9.

23. Parasuraman S, Raveendran R, Madhavrao C. GC-MS analysis of leaf extracts of CleistanthuscollinusRoxb. (Euphorbiaceae). Int J Pharm Sci 2009;1(2):284-6.

24. Parekh, J., &Chanda, S. (2007). In vitro antimicrobial activity and phytochemical analysis of some Indian medicinal plants. *Turkish journal of biology*, *31*(1), 53-58.

25. Prashith, K. T. R., Vivek, M. N., Junaid, S., Rakesh, K. N., Dileep, N., Manasa, M., &Kambar, Y. (2014). Inhibitory activity of Polyalthialongifolia, Anaphalislawii and Gnidia glauca against Colletotrichumcapsici and urinary tract pathogens. *Science, Technology and Arts Research Journal*, *3*(1), 26-30.

26. Praveen KP, Kumaravel S, Lalitha C. Screening of antioxidant activity, total phenolics and GC-MS study of Vitexnegundo. African J Biochemistry Res 2010;4(7):191-5.

27. Rao, S. B., Jayanthi, M., Yogeetha, R., Ramakrishnaiah, H., &Nataraj, J. (2013). Free radical scavenging activity and reducing power of Gnidia glauca (Fresen.) Gilg. *Journal of Applied Pharmaceutical Science*, *3*(6), 203-207.

28. Rastogi N, Moreau B, Capmau ML, Goh KS, David HL. Antibacterial action of amphiphatic derivatives of isoniazid against the Mycobacterium avium complex. ZentralblattfürBakteriologie, Mikrobiologie und Hygiene 1988;268:456–62.

29. Senthilkumar N, Murugesan S, Vijayalakshmi KB. C-MS-MS analysis of Trichiliaconnaroides (Wight &Arn.) Bentv (Meliaceae): A tree of ethnobotanical records. Asian J Plant Sci Res 2012;2(2):193-7. Ed. St. Paul: Cengage Learning, Brooks/Cole Publishing Co; 2006.

30. Sermakkani M, Thangapandian V. GC-MS analysis of Cassia italica leaf methanol extract. Asian J Pharm Clinical Res 2012;5(2):90-4.

31. Sharon, M., & Gupta, A. (2009). In vitro culture of Lasiosiponeriocephalusan Endangered Species. *Academic Journal of Plant Sciences*, *2*(2), 92-96.

32. Shewale, V. S., Khairmode, P. V., Lawand, S. T., Netam, V. S., &Bhosale, A. R. (2022). Antifeedant and Insecticidal Activity of Different Solvent Extracts of *Vitex negundo* (L) against Cotton Leafworm Spodoptera litura (Fab.). *Asian Journal of Research in Zoology*, *5*(4), 32-38.

33. Subashri B, Pillai YJ. A comparative study of antioxidant activity of Baccopamonnieri(L.) Pennell using various solvent extracts and its GC-MS analysis. Int J Pharm PharmSci 2014;6(2): 494-8.

34. Teklehaymanot, T., & Giday, M. (2007). Ethnobotanical study of medicinal plants used by people in Zegie Peninsula, Northwestern Ethiopia. *Journal of ethnobiology and Ethnomedicine*, *3*(1), 1-11.

35. Trabalon M, Niogret J, Legrand-Frossi C. Effect of 20- hydroxyecdysone on cannibalism, sexual behavior, and contact sex pheromone in the solitary female spider, Tegenariaatrica. General Comparative Endocrinology 2005;144:60–6.

36. Van Andel, T. (2000). The diverse uses of fish-poison plants in Northwest Guyana. *Economic Botany*, 500-512.

37. Wabale, A. S., &Kharde, M. N. (2010). Effect of plant extracts on the fungal pathogen causing wilt of sugarcane in in vitro. *International Journal of Plant Sciences (Muzaffarnagar)*, *5*(2), 445-447.

38. Walker K, Croteau R. Taxol biosynthesis: molecular cloning of a benzoyl-CoA: taxane 2a-O-benzoyltransferase cDNA from Taxus and functional expression in Escherichia coli. Proceedings of the National Academy of Sciences of the United States of America 2000; 97:13591–6.

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Advances in SMA: Genetic Insights, Prevalence in GCC, and Emerging Therapeutic Approaches

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Abstract

SMA is a neurodegenerative autosomal recessive disorder characterized by progressive degeneration of motor neurons of the spinal cord, leading to muscle weakness and early mortality. SMA can be divided into four types, extending from severe infantile-onset type I to milder forms that appear later in life, such as types II, III, and IV. The GCC countries have an unusually high prevalence of SMA, largely due to the high rate of consanguineous marriages, a situation that increases the risk of inheritance of autosomal recessive conditions. Genetic predisposition to SMA is due to mutations in the Survival Motor Neuron gene occurring in two almost identical copies, SMN1 and SMN2. The severity of this disease is correlated with the number of functional copies of these genes; however, SMN2 is also capable of playing a very critical compensatory role. The more the number of copies of SMN2 one has, the milder the forms of the disease. Molecular screening techniques such as genetic testing and carrier screening are very essential in this regard for early diagnosis and management of SMA. This orphan disease has several FDA- and EMA-approved treatment options for SMA, including Nusinersen, Onasemnogene abeparvovec, and Risdiplam. Results with these treatments are very promising and show improved motor function and survival in the affected patients. The present review outlines current developments in research and treatment of the disease, drawing attention to the high prevalence of the condition in GCC countries and also to the contribution that genetic screening, combined with the emerging therapies, can have in managing this devastating disorder.

Keywords: SMA, SMN1, SMN2, Risdiplam, Onasemnogene Abeparvovec, Nusinersen

Introduction

Spinal muscular atrophy (SMA) includes a group of inherited neurodegenerative diseases of an autosomal recessive nature. Clinically, SMA is characterized by muscle weakness and atrophy associated with loss of spinal and sometimes bulbar motor neurons (1).

Since the disease's first clinical identification in 1891, we have learned more about its pathogenesis and anatomical features during the course of the following century (1, 2, 3, 4). This ailment was first linked to the 5q13.2 locus in 1990, and by 1995, the gene survival motor neuron (SMN) was implicated in the disorder's presentation (1, 4). The identification of the gene helped in the creation of animal models by the early 2000's, and this in turn enabled the identification of the role of *SMN2* in preserving the phenotype and providing new avenues of therapeutic interventions. Advances in SMA genetic testing and diagnosis have provided accurate epidemiological information; at least 1 in 10,000–20,000 live newborns have the condition, and over 90% of affected individuals are homozygous for SMN1 deletion (5; 4). In contrast, some studies report that SMA affects approximately 1 in 6,000 individuals and is the most common form of hereditary cause of childhood mortality (2).

This review gleans insights and evidence from published literature and aims to provide a comprehensive overview of the condition, mainly focusing on SMA's genetic predisposition, global and national frequencies in Saudi Arabia, and molecular screening and testing techniques.

Clinical manifestations

Classically, SMA presents with progressive muscle weakness and atrophy leading to irreversible loss of anterior horn cells in the spinal cord and the brain stem nuclei. The weakness associated with the condition ranges, with the weakness being progressive, symmetric, and greater in the proximal region than in the distal. Since its identification, attempts have been made to classify SMA into discrete and identifiable phenotypic subtypes, but with the advent of diagnostic technologies, it has become clear that SMA has varied phenotypes (6). Advances in next-generation technologies have now identified as many as 33 genes that have been implicated in the manifestation of the SMA phenotype. This technology has enabled researchers to identify the mechanisms that include defects in RNA metabolism and splicing, axonal transport, and motor neuron development and connectivity (6). This unraveling of mechanisms has helped progress from the disease being referred to as SMA5q (the most common form of the condition) to a multitude of phenotypes that can be attributed to the genetic heterogeneity (6). The location of the gene (*SMN1*) responsible for SMA is a complex locus, as it contains a 500-kb duplication and loss of a telomeric region, which is what leads to SMA, while the *SMN2* gene is a centromeric gene. Both genes differ by one exon, i.e., exon 7, that is spliced out in the *SMN2* gene because of a C>T transition at c840. Though most of the mRNA transcripts produced do not contain exon 7, there still are some transcripts that escape this predicament, and these transcripts are responsible for the 5-10% of full-length transcripts that rescue the SMN1 phenotype (7). A multitude of patients (approximately 96%) present with a *SMN1* homozygous deletion for the exons seven and eight or exon seven only, whereas the other four percent are compound heterozygotes for the *SMN1* locus with a deletion of one allele and a point mutation at the other (8).

The most common form of the condition manifests due to the homozygous disrupted *SMN1,* which results in a reduction in the SMN levels in the motor neurons, and is an autosomal recessive condition with a frequency of 1 in 6000 to 10,000 and a carrier frequency of 1 in 40 to 60 adults (6). The latest technological innovations have now been able to classify SMA into four types according to their symptom severity and genotypes, though this might change based on the advancements that have been achieved in the treatment of the condition (9). The below-given table classifies the different types of SMA (Table 1) (6).

Table 1: Spinal muscular atrophies (SMA) with known gene abnormalities/loci (adopted from Farrar & Kiernan, 2015)

Type of SMA	Inheritance	Age of onset	Clinical Phenotype	Gene/ Locus	Gene identified in
Proximal SMA SMA5q or SMN-related SMA	Autosomal recessive	By six months	Proximal greater than distal limb weakness; diaphragm and facial muscles relatively spread	SMN1	1995
SMA Type A (infantile SMA, Werdnig- Hoffman disease)					
SMA Type 2					
SMA Type 3 (Kugelberg-Welander disease)		6-18 months			
		18 months			
SMA Type 4		Adult			
X-linked infantile SMA Type 2	X-linked	Infantile	Similar to SMA Type 1. Severe congenital hypotonia, arthrogryposis	UBE1	2008
SMA phenotype due to mitochondrial dysfunction	Autosomal recessive	Infantile	Similar to SMA Type 1 with dilated cardiomyopathy, ptosis, impaired extraocular movements.	SCO2	1999
SMA with pontocerebellar hypoplasia type 1	Autosomal recessive	Congenital or infantile	Diffuse weakness, microcephaly with/without arthrogryposis	EXOSC3 TSEN54 RARS2 VRK1	2012 2011 2011 2009
SMALED1	Autosomal dominant	Congenital to adult	Proximal greater than distal leg weakness, arms normal	DYNC1H1	2012
SMALED2	Autosomal dominant	Congenital to adult	Proximal and distal muscle weakness of the lower limbs with or without mild upper limb weakness, mild upper motor signs	BICD2	2013
DSMA4	Autosomal recessive	By three years	Proximal weakness, difficulty walking and climbing stairs, progressing to nonambulant and respiratory weakness.	PLEKHG5	2007

Adult-onset proximal SMA	Autosomal dominant	Adult	Proximal greater than distal weakness	VAPB	2004
Adult-onset proximal SMA followed by cardiac involvement	Autosomal dominant	Adult	Proximal greater than distal weakness	LMNA	2007
Adult-onset proximal SMA with respiratory failure	Autosomal dominant	Adult	Proximal weakness of upper limbs followed by prominent respiratory failure	MAPT	2014
Spinal and bulbar muscular atrophy (Kennedy Syndrome)	X-linked recessive	Adult	Widespread and prominent fasciculations, Progressive proximal and distal limb and bulbar muscle weakness and atrophy, dysphagia, gynaecomastia, and androgen resistance	AR	1991
Distal SMA/HMN					
HMN1	Autosomal dominant	Child to young adult	Distal leg then arm weakness	HSPB1, HSPB8, GARS, DYNC1H1, 7q34	
HMN2	Autosomal dominant	Adult	Distal leg then arm weakness		
HMN2A				HSPB8	2004
HMN2B				HSPB1	2004
HMN2C				HSPN3	2010
HMN2D				FBXO38	2013
HMN3 (DSMA3)	Autosomal recessive	Childhood	Mild distal leg then arm weakness	11q13	
HMN4 (DSMA3)	Autosomal recessive	Infancy to young adult	Severe proximal and distal weakness, diaphragmatic palsy	11q13	
HMN5	Autosomal dominant	Child to adult	Upper limb predominance with onset in thenar and first dorsal interosseus muscles and subsequent weakness of legs		
HMN5A				GARS	2003
HMN5B				REEP1	2012
HMN5C				BSCL2	2004
HMN6 (DSMA1 or SMARD1)	Autosomal recessive	Infancy	Early diaphragm weakness, distal greater than proximal limb weakness	IGHMBP2	2001

HMN7 HMN7A HMN7B	Autosomal dominant	Juvenile/ young adult	Vocal cord paresis, hand weakness and subsequent distal leg weakness	SLC5A7; CHT DCTN1	2012 2003
DSMA5	Autosomal recessive	Young adult	Progressive distal greater than proximal lower limb muscle weakness and atrophy	DNAJB2; HSJ1	2012
HMN with upper motor neuron signs	Autosomal dominant	Juvenile	Distal leg then arm weakness with pyramidal signs	SETX	2004
HMNJ (DSMA2)	Autosomal recessive	Juvenile	Distal leg then arm weakness with pyramidal signs originating from the Jerash region of Jordan	9p21	2000
X-linked dHMN (SMAX3)	X-linked recessive	Child to adult	Distal leg then arm weakness	ATP7A	2010
SMARD2	X-linked recessive	Neonatal	Distal weakness, early onset diaphragmatic weakness and respiratory failure	LASIL	2014
		Young adult	Distal and scapuloperoneal weakness with or without congenital absence of muscles or laryngeal palsy	TRPV4	2010
Congenital DSMA	Autosomal dominant	Congenital	Proximal and distal non-progressive lower limb weakness with or without vocal cord paralysis or arthrogryposis	TRPV4	2010
DSMA with mitochondrial dysfunction	Mitochondrial	Childhood to adult	Episodic weakness associated with a later-onset distal lower limb weakness and atrophy Episodic weakness associated with a later-onset distal motor neuropathy	mtATP6 mtATP8	2012 2013

*Distal HMN is based on the original classification by Harding

SMN: survival motor neuron; SMALED: spinal muscular atrophy with lower extremity predominance; DSMA: distal spinal muscular atrophy; HMN: distal hereditary motor neuropathy, SMARD: spinal muscular atrophy with respiratory distress, HMNJ: distal hereditary motor meuropathy, Jerash type; dHMN: distal HMN

Most 5q SMA patients present with a homozygous deletion of the seventh exon of *SMN1,* with the remaining presenting with a deletion and a heterozygous point mutation. De novo mutations have been reported in two percent of the cases, indicating that this particular region on the fifth chromosome is unstable due to the presence of a high number of copy number repeats causing problems during the crossing over, thus leading to de novo mutations. Patients who present with mild symptoms might undergo gene conversion of the *SMN1* and *SMN2* genes (10). The number of copies of SMN2 varies in the general population, with 15% of individuals not having even a single copy of the gene, 33% having a single copy, and 50% of the general population having two copies of the gene (10).

Spinal muscular atrophy in gulf cooperation council countries

Data specific to SMA in GCC is limited, but there is an increase in it. Research shows that globally, the occurrence of SMA is different, with estimates ranging from 1 case per 6,000 live births. Caused by genetic factors and consanguinity, there are high

levels of this illness within some populations that could be higher than others. The number of cases of genetic abnormalities such as SMA could be on the rise in GCC countries than in non-consanguineous communities, as consanguineous marriages are more common.

"In the GCC states the distribution and prevalence of SMA genotypes are influenced by a number of factors, consisting of consanguinity rates inclusive of genetic diversity within populations of Saudi Arabia, Kuwait, Qatar, Bahrain, and Oman, along with the United Arab Emirates." According to this quotation from Alkuraya et al. (2004), approximately 20% of loci known so far exhibit no polymorphisms, while most others depict variability along their DNA sequence, such as insertion/deletions, frameshifts, etc. Among them, it was found that ~42% came within a 100-bp region near one neighboring coding gene (11)."

Saudi Arabia has a very high prevalence of SMA, estimated at 13.26 per 100,000 people (12; 13). The incidence rate is equally high, estimated at 40 times the rate in Western countries at 10-193 for every 100,000 births (14). Verhaart et al. (2017) attribute the high prevalence to parental consanguinity (i.e., intra-familial unions involving first/second cousins or other familial ties). Studies estimate that 1 billion people reside in communities that practice consanguineous marriages, especially in the Middle East, North Africa, and West Asia (15). An estimated 20% to 50% of marriages worldwide are considered to be such marriages. As a result, children born in these areas have a high incidence of genetic and congenital illnesses. The closer the familial relation, the higher the risk of the offspring inheriting identical copies of genes (including faulty ones) from both parents. A recent study in Saudi Arabia reported an SMA carrier frequency of 2.6%, which is higher than the reported global frequency of 1.25–2% (16). Valuable insights into the genetic landscape of SMA have been provided by research undertaken in Saudi Arabia. In the study carried out by Alfadhel et al. (2017), they investigated the molecular genetics of SMA in a group of patients from this country. The prevalent type of SMA among all affected individuals participating in their research turned out to be possession of one copy only of one gene called SMN1 exon 7, which resulted in its complete deletion on both chromosomes, resulting in what is known as homozygous deletions. Thereafter signs emerged as those of classical nerve degeneration, which involved early age onset while leaving individuals fully restrained or even dead (17). Studies that have explored Kuwait's genetic epidemiology of SMA have provided insight into its prevalence and the specific genetic variants that are present in this population. The disorder is seen among different ethnic and cultural groups across Kuwait, although there has been no mention of particular incidences of SMA in the literature (18).

Diagnosis of spinal muscular atrophy

Diagnosing SMA primarily depends on the deletion of the SMN1 gene and the number of SMN2 gene copies, which are prognostic indicators and modifiers of the disease. Early onset of SMA is linked to fewer SMN2 copies, resulting in shorter survival, whereas more copies correlate with later onset and longer survival (19). Phosphorylated neurofilament chains also serve as biomarkers, with elevated levels observed in presymptomatic individuals with two SMN2 copies and in SMA patients (20; 21). Non-molecular biomarkers like compound muscle action potential (CMAP) amplitude, motor unit number estimation (MUNE), and electrical impedance myography (EIM) are crucial for understanding SMA's etiopathogenesis and differentiating between symptomatic and presymptomatic patients (20). Imaging tools such as MRI, muscle ultrasound, and multispectral optoacoustic tomography help detect the symptomatic phase of SMA (19).

Genetic counselling in spinal muscular atrophy

Genetic counseling plays a vital role

in identifying individuals at risk of inheriting diseases or passing on the risk alleles to the future generation. Generally, prenatal testing is requested by would-be parents who have had prior history of the condition in their family or have a child who is positive for SMA. Newborn screening should be made compulsory in those families that have a history of SMA within their family. Because most cases arise from consanguineous marriages, it is critical that the couple undertake genetic testing to avoid complications.

Treatment for spinal muscular atrophy

Treatment options for spinal muscular atrophy (SMA) are under continuous exploration due to the rarity of the condition. This scarcity has led to limited interest from drug manufacturers in developing SMA therapies. Currently, the available treatments are orphan drugs, which are specifically developed for rare diseases. Three orphan drugs have been authorized for SMA treatment: nusinersen, onasemnogene abeparvovec, and risdiplam (22).

Nusinersen

This antisense oligonucleotide was the first drug approved for SMA by the FDA in 2016 and the EMA in 2017. Nusinersen targets the intronic splice silencing site (ISS-N1) in intron 7 of the SMN2 gene, preventing the binding of hnRNPA1 and hnRNPA2 to ISS-N1. This allows exon 7 to be included in the final transcript, producing a full-length SMN protein. However, nusinersen cannot cross the blood-brain barrier, requiring recurrent lumbar punctures for administration (22).

Onasemnogene abeparvovec

Approved in 2020 for patients under two years old with biallelic SMN1 mutations and either three or four copies of the SMN2 gene or infantile-onset SMA, this gene replacement therapy uses adeno-associated viral vector serotype 9 (scAAV9) for delivery. It works through recombinant self-complementation and has shown significantly improved lifespan in preclinical mouse studies, with the ability to cross the blood-brain barrier (22). This suggests potential effectiveness in humans diagnosed early in life.

Risdiplam

An oral medication approved in 2020 by the FDA and EMA, risdiplam is a coumarin-based pyrido-pyrimidinone derivative. It has excellent tissue distribution, as shown in pharmacokinetic analyses from preclinical studies (22). These advancements in SMA treatment highlight the ongoing efforts to manage this rare but debilitating condition.

Conclusion

Spinal muscular atrophy (SMA) is a genetic disability marked by progressive muscle degeneration and diminished strength due to the breakdown of nerve cells controlling muscle movement in the brainstem and spinal cord. This condition primarily results from SMN1 gene mutations, with varying severity levels from type 0 (detected before birth) to type IV (appearing in adulthood). The disorder severely impacts patients' mobility, affecting activities like walking and sitting, and can lead to breathing and swallowing difficulties in advanced stages. Early identification and management are crucial. Significant advancements in genetic research and therapeutics, including treatments like nusinersen (Spinraza), onasemnogene abeparvovec (Zolgensma), and risdiplam (Evrysdi), have improved the outlook for SMA patients by potentially halting or restoring neurological functions. Timely initiation of these treatments, supported by newborn screening programs, is essential. Thus, further efforts are still being made in relation to gene therapy, while other complementary treatments, such as physiotherapy/occupational therapy, ventilator use, and nutrition management, have led to better standards of living among patients with SMA. However, even with advancements, some problems still exist. High treatment costs, lifelong need for medical attention,

and their effect on the social and mental well-being of patients and their relatives require that management be all-inclusive. To address these demands, joint health workers must have multidisciplinary teams comprising neurologists, genetic counselors, and others. Continued research, early intervention, and holistic care approaches are key to improving outcomes and offering hope to individuals and families affected by this disorder.

Conflict of Interest

All the authors state that none of them have any conflict of interest.

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References

1. Sumner, C.J., Paushkin, S. and Ko, C.-P. (2017) *Spinal muscular atrophy: Disease mechanisms and therapy*. London: Academic Press, an imprint of Elsevier.

2. Ross, L.F. and Kwon, J.M. (2019) 'Spinal muscular atrophy: Past, present, and future', *NeoReviews*, 20(8). doi:10.1542/neo.20-8-e437.

3. Mercuri, E. *et al.* (2022) 'Spinal muscular atrophy', *Nature Reviews Disease Primers*, 8(1). doi:10.1038/s41572-022-00380-8.

4. Nishio, H. *et al.* (2023) 'Spinal muscular atrophy: The past, present, and future of diagnosis and treatment', *International Journal of Molecular Sciences*, 24(15), p. 11939. doi:10.3390/ijms241511939.

5. Kolb, S.J. and Kissel, J.T. (2015) 'Spinal muscular atrophy', *Neurologic Clinics*, 33(4), pp. 831–846. doi:10.1016/j.ncl.2015.07.004.

6. Farrar, M.A. and Kiernan, M.C. (2015) 'The genetics of Spinal Muscular Atrophy: Progress and challenges', *Neurotherapeutics*, 12(2), pp. 290–302. doi:10.1007/s13311-014-0314-x.

7. Mailman, M.D. *et al.* (2002) 'Molecular analysis of spinal muscular atrophy and modification of the phenotype by SMN2', *Genetics in Medicine*, 4(1), pp. 20–26. doi:10.1097/00125817-200201000-00004.

8. Alías, L. *et al.* (2008) 'Mutation update of spinal muscular atrophy in Spain: Molecular characterization of 745 unrelated patients and identification of four novel mutations in the SMN1 gene', *Human Genetics*, 125(1), pp. 29–39. doi:10.1007/s00439-008-0598-1.

9. Keinath, M.C., Prior, D.E. and Prior, T.W. (2021) 'Spinal muscular atrophy: Mutations, testing, and clinical relevance', *The Application of Clinical Genetics*, Volume 14, pp. 11–25. doi:10.2147/tacg.s239603.

10. Oskoui, M., Darras, B.T. and De Vivo, D.C. (2017) 'Spinal muscular atrophy', *Spinal Muscular Atrophy*, pp. 3–19. doi:10.1016/b978-0-12-803685-3.00001-x.

11. Rabea, F. *et al.* (2024) 'Spinal muscular atrophy genetic epidemiology and the case for premarital genomic screening in Arab populations', *Communications Medicine*, 4(1). doi:10.1038/s43856-024-00548-1.

12. Verhaart, I.E. *et al.* (2017) 'Prevalence, incidence and carrier frequency of 5q–linked spinal muscular atrophy – a literature review', *Orphanet Journal of Rare Diseases*, 12(1). doi:10.1186/s13023-017-0671-8.

13. Al Jumah, M. *et al.* (2022) 'Spinal muscular atrophy carrier frequency in Saudi Arabia', *Molecular Genetics & Genomic Medicine*, 10(11). doi:10.1002/mgg3.2049.

14. Alghamdi, A. *et al.* (2023) 'Identifying clinical and genetic characteristics of spinal muscular atrophy patients and families in Saudi Arabia', *Cureus* [Preprint]. doi:10.7759/cureus.46452.

15. Hamamy, H. (2011) 'Consanguineous marriages', *Journal of Community Genetics*, 3(3), pp. 185–192. doi:10.1007/s12687-011-0072-y.

16. AlTawari, A. *et al.* (2024) 'Nusinersen treatment for spinal muscular atrophy: Retrospective multicenter study of pediatric and adult patients in Kuwait', *Neurology International*, 16(3), pp. 631–642. doi:10.3390/neurolint16030047.

17. Alfadhel, M. *et al.* (2017) 'Expanded newborn screening program in Saudi Arabia: Incidence of screened disorders', *Journal of Paediatrics and Child Health*, 53(6), pp. 585–591. doi:10.1111/jpc.13469.

18. Mendell, J.R. *et al.* (2017) 'Single-dose gene-replacement therapy for spinal muscular atrophy', *New England Journal of Medicine*, 377(18), pp. 1713–1722. doi:10.1056/nejmoa1706198.

19. Babić, M. *et al.* (2023) 'Molecular biomarkers for the diagnosis, prognosis, and pharmacodynamics of spinal muscular atrophy', *Journal of Clinical Medicine*, 12(15), p. 5060. doi:10.3390/jcm12155060.

20. Pino, M.G., Rich, K.A. and Kolb, S.J. (2021) 'Update on biomarkers in spinal muscular atrophy', *Biomarker Insights*, 16, p. 117727192110356. doi:10.1177/11772719211035643.

21. Schwartz, O. *et al.* (2022) 'Spinal muscular atrophy – is newborn screening too late for children with two SMN2 copies?', *Journal of Neuromuscular Diseases*, 9(3), pp. 389–396. doi:10.3233/jnd-220789.

22. Crisafulli, S. *et al.* (2023) 'Pharmacological therapies of spinal muscular atrophy: A narrative review of Preclinical, clinical–experimental, and real-world evidence', *Brain Sciences*, 13(10), p. 1446. doi:10.3390/brainsci13101446.

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BCL Protein and It's Intrinsic Apoptotic Pathway: A Literature Review

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Abstract:

The BCL (B-cell lymphoma) protein family is a diverse group of regulators critically involved in apoptosis, a tightly controlled process crucial for maintaining tissue homeostasis. Dysregulation of apoptosis is a common feature in cancer, emphasizing the need to comprehend the intricate mechanisms underlying the function of the BCL family. This literature review offers a comprehensive exploration of the structural domain organization of the BCL family, shedding light on how these proteins orchestrate apoptosis. The family comprises anti-apoptotic proteins, pro-apoptotic effectors, and BH3(BCL-2 homology 3)-only proteins, each with distinct structural features and functional roles in apoptotic signaling pathways. Anti-apoptotic proteins like BCL-2 serve as guardians against cell death by preserving mitochondrial integrity. Pro-apoptotic effectors, on the other hand, actively promote apoptosis by inducing mitochondrial outer membrane permeabilization. BH3-only proteins act as molecular switches, mediating the delicate balance between pro- and anti-apoptotic signals. This review delves into the specific role of BCL-2, a prominent family member, in various cancers such as lung, breast, and prostate cancers. Highlighting its significance as a potential therapeutic target, the article underscores the importance of understanding the molecular nuances of BCL-2 in cancer progression. Moreover, recent advancements in BCL-2 inhibitor development are discussed, showcasing their potential as targeted therapies for cancer treatment. These inhibitors represent a promising avenue for personalized cancer therapy, aiming to selectively induce apoptosis in cancer cells while sparing normal cells. The review emphasizes the importance of these inhibitors in addressing the specific challenges posed by BCL-2 dysregulation in diverse cancer types. By elucidating the structural and functional aspects of the BCL protein family, this literature review provides valuable insights into apoptotic signaling pathways. It not only deepens our understanding of the molecular intricacies governing cell death but also presents novel strategies to modulate apoptosis in cancer cells. Ultimately, the article highlights the significance of BCL proteins as promising therapeutic targets and the potential of BCL-2 inhibitors for personalized cancer therapy, paving the way for advancements in cancer treatment.

Key Words: BCL-2 proteins, BH3 only proteins, anti-apoptotic proteins, pro-apoptotic proteins

Introduction

The B-cell lymphoma (BCL) protein family encompasses a diverse group of regulatory proteins involved in the intricate control of apoptosis, a fundamental process in cellular

homeostasis (1). Apoptosis, or programmed cell death, plays a crucial role in embryonic development, tissue remodelling, and the elimination of damaged or infected cells(2). Dysregulation of apoptosis is strongly associated with the pathogenesis of various diseases, including cancer, Studying of BCL proteins essential for understanding and developing therapeutic strategies (2).

The BCL family can be classified (Figure 1) into three main groups based on their function and structural domains: anti-apoptotic proteins (e.g., BCL-2, BCL-XL), pro-apoptotic effectors (e.g., BAX, BAK), and BCL-2 homology-3 (BH3) only proteins (e.g., BIM, BAD)(3). These proteins dynamically interact and regulate the balance between cell survival and death by modulating mitochondrial integrity, caspase activation, and the release of pro-apoptotic factors (4).

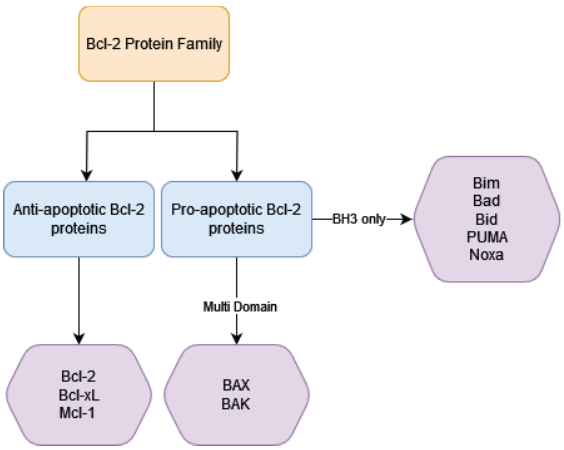

Figure 1: Classification of Bcl-2 Protein Family

Overexpression of B-cell lymphoma 2 (BCL-2) has been associated with increased resistance to apoptosis, allowing cells to evade programmed cell death and contribute to tumorigenesis and cancer progression (4). The dysregulation of BCL-2 expression or function can lead to the evasion of apoptosis, promoting cell survival, tumor growth, and resistance to therapeutic interventions. Therefore, understanding the role of apoptosis in regulating the BCL-2 protein is important in cancer research and may provide valuable insights into the development of targeted therapies (4).

Investigating the role of BCL proteins in cancer research is of paramount importance due to their central involvement in apoptosis and their dysregulation in various malignancies (5). Aberrant expression of BCL proteins, such as upregulation of anti-apoptotic proteins or loss of pro-apoptotic effectors, can confer a survival advantage to cancer cells, contributing to tumor initiation, progression, and therapy resistance (6).

Recent studies have highlighted the clinical significance of BCL proteins as diagnostic and prognostic markers in multiple cancer types (7). For instance, BCL-2 overexpression has been associated with adverse outcomes in lung cancer and breast cancer while BCL-2 downregulation has been linked to aggressive prostate cancer phenotypes (8). Understanding the structural and functional aspects of BCL proteins can uncover novel therapeutic targets and inform the development of precision medicine approaches.

One of the remarkable advancements in the field has been the development and utilization of small-molecule inhibitors and BCL-2 homology 3 (BH3) mimetics that specifically target the BCL-2 protein (9). These compounds aim to disrupt the interactions between BCL-2 and its pro-apoptotic counterparts, such as BCL-2-associated X protein (BAX) and BCL-2 homologous antagonist/killer (BAK) and restore the apoptotic signalling cascade (9,10). By inhibiting the anti-apoptotic function of BCL-2, these drugs promote apoptosis and sensitize cancer cells to cytotoxic therapies (11).

Venetoclax (ABT-199), a potent BCL-2 inhibitor, has emerged as a breakthrough therapeutic agent in the treatment of certain haematological malignancies (12). It has demonstrated remarkable efficacy as a single agent or in combination with other chemotherapeutic drugs in various haematological cancers, including

chronic lymphocytic leukaemia (CLL) and acute myeloid leukaemia (AML) (12,13). Venetoclax has been approved by regulatory authorities and is commercially available for the treatment of specific indications. Other BCL-2 inhibitors currently under investigation include navitoclax (ABT-263) and sabutoclax (BI-97C1), which target both BCL-2 and related anti-apoptotic proteins, such as B-cell lymphoma-extra-large (BCL-XL) and myeloid cell leukaemia sequence 1 (MCL-1) (13). These inhibitors have shown promising results in preclinical studies and early-phase clinical trials, demonstrating their potential as therapeutic options for various cancers (12).

Furthermore, the development of BH3 mimetics, which mimic the BH3 domain of pro-apoptotic proteins and selectively target anti-apoptotic proteins, has opened new avenues for precision medicine (14). These BH3 mimetics, such as obatoclax, ABT-737, and ABT-199, have shown efficacy in preclinical models and are being evaluated in clinical trials for the treatment of haematological and solid malignancies (15).

Moreover, the development of small-molecule inhibitors and BH3 mimetics that selectively target BCL proteins has shown promising results in preclinical and clinical studies. These targeted therapies aim to restore the delicate balance between pro- and anti-apoptotic BCL proteins, sensitizing cancer cells to cell death and overcoming treatment resistance (15). Therefore, unravelling the intricate mechanisms of BCL protein regulation and their interactions provides a foundation for the development of innovative therapeutic strategies in cancer treatment.

Structural domain of BCL family

The BCL-2 homology (BH) domains constitute a crucial structural feature of the BCL protein family. BH domains are conserved regions within the protein sequence that participate in protein-protein interactions and determine the functional properties of BCL proteins (16). The BH domains are classified into four subgroups: BH1, BH2, BH3, and BH4. Each subgroup has distinct structural and functional characteristics. BH1 and BH2 domains are primarily found in the multi-domain anti-apoptotic proteins, while BH3 domains are present in both pro-apoptotic effectors and BH3-only proteins (16). Multi-domain anti-apoptotic proteins, such as BCL-2, BCL-XL, and MCL-1, contain multiple BH domains, including BH1, BH2, and BH3 (17).

Understanding the structural organization of BCL proteins and their functional domains is important in elucidating the complex mechanisms underlying apoptosis. Dysregulation of BCL proteins can lead to abnormal cell survival, contributing to the development and progression of various diseases, including cancer (18).

Anti-apoptotic Proteins

BCL-2

BCL-2 interacts with proteins like BAX and BAK, which promote cell death. BCL-2 forms complexes with these proteins, preventing them from causing damage to the mitochondria and stopping the release of apoptotic factors (19). BH3-only proteins such as BAD, BIM, PUMA, and BID can neutralize the inhibitory effect of BCL-2, allowing other pro-death proteins to initiate apoptosis. Additionally, there are synthetic compounds called BH3 mimetics that mimic the function of BH3-only proteins. BH3 mimetics can disrupt the interaction between BCL-2 and pro-survival proteins, leading to apoptosis (19).

BCL-XL

BCL-XL (BCL2L1) protein consists of structural domains including BH1, BH2, and BH3 domains (20). These domains are important for protein-protein interactions and modulating the function of BCL-XL. BCL-XL is primarily localized in the mitochondria, where it exerts its anti-apoptotic effects (21). By residing in the outer membrane of mitochondria, BCL-XL

protects the integrity of these organelles and prevents the release of apoptotic factors. Interacting proteins of BCL-XL include Bax and Bak, which are pro-apoptotic proteins (21). BCL-XL forms heterodimers with Bax and Bak, inhibiting their pro-apoptotic activity (21). This interaction prevents the formation of pores in the mitochondrial membrane, thereby blocking the release of apoptotic factors. Key regulators of BCL-XL include BH3-only proteins like Bim, Bad, and Puma (22). These proteins can bind to BCL-XL and neutralize its anti-apoptotic function, allowing pro-apoptotic proteins to promote apoptosis (22,23). Recent studies have shed light on the importance of BCL-XL in cell survival and apoptosis regulation (23). A study presented a case of a patient with metastatic colon cancer and highlighted the role of BCL-XL in the progression of the disease. The study found that the patient had a mutation in the v-Raf murine sarcoma viral oncogene homolog B (BRAF) and a deficiency in mismatch repair, which led to the overexpression of BCL-XL and contributed to the development of metastatic lesions (23).

MCL-1

MCL-1 possesses structural domains including BH1, BH2, and BH3 domains. These domains are important for its interactions with other proteins and modulating its anti-apoptotic function (24). The protein is primarily localized in the mitochondria, where it exerts its anti-apoptotic effects (25). By residing in the outer membrane of mitochondria, MCL-1 helps maintain mitochondrial integrity and prevents the release of apoptotic factors. MCL-1 interacts with several proteins, including pro-apoptotic members of the BCL-2 family such as BIM, BAK, and NOXA (26). These interactions are crucial for determining the balance between cell survival and apoptosis. MCL-1 can form heterodimers with pro-apoptotic proteins, inhibiting their ability to promote apoptosis (27).

BCL-W

BCL-W (BCL2L2) is an anti-apoptotic protein that has been less studied compared to other members of the BCL-2 family (28). It shares structural homology with other anti-apoptotic proteins, including BCL-2 and BCL-XL. Like other BCL-2 family members, BCL-W functions to inhibit apoptosis and promote cell survival (28).

BFL-1

This protein consists of BH1, BH2, and transmembrane domains, which are essential for its interactions with other proteins and its anti-apoptotic function. BFL-1 is primarily localized to the mitochondria, particularly the outer mitochondrial membrane (29). BFL-1 has been associated with various cellular processes and interactions. One notable interaction involves its binding to Beclin-1, which enhances the proliferation of macrophages and mast cells during allergic reactions. By interacting with Beclin-1, BFL-1 supports the survival and expansion of these immune cells (30).

Pro-apoptotic Effectors

BAX (Bcl-2-associated X protein)

BAX is a pro-apoptotic protein that plays a crucial role in programmed cell death. It is activated by various apoptotic signals, such as DNA damage, endoplasmic reticulum stress, and growth factor withdrawal (31). The activation of BAX involves a series of conformational changes, oligomerization, and membrane insertion (32). These processes lead to the formation of pores in the outer mitochondrial membrane, known as mitochondrial outer membrane permeabilization (MOMP) (31). As a result, Cytochrome c, among other factors, is released into the cytoplasm, leading to the assembly of the apoptosome and subsequent activation of caspases, initiating cell death (33).

BAK (Bcl-2 antagonist/killer)

Like BAX, BAK interacts with anti-apoptotic proteins such as BCL-2 and BCL-XL. The dynamic balance between BAK and these anti-apoptotic proteins determines whether apoptosis is initiated or prevented (34).

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BOK (Bcl-2-related ovarian killer)

BOK exhibits a broader range of pro-apoptotic activities compared to BAX and BAK. It can induce apoptosis through both mitochondrial-dependent and mitochondrial-independent pathways (35). BOK interacts with Beclin-1 and other autophagy-related proteins, modulating autophagic activity (36). BOK expression is regulated by cellular stressors such as DNA damage and viral infection. Its upregulation can contribute to cell death in response to these stressors (36).

BH3- Domain only Protein

Bcl-2-interacting mediator of cell death (BIM)

The binding of BIM to anti-apoptotic proteins results in the displacement of pro-apoptotic effectors, leading to their activation and subsequent initiation of apoptosis (37). BIM can also directly activate BAX and BAK by interacting with their hydrophobic grooves, promoting their oligomerization and pore formation in the mitochondrial outer membrane (38).

BH3-interacting domain death agonist (BID)

BID serves as a critical link between the extrinsic and intrinsic apoptotic pathways (Figure 2), connecting death receptor activation to mitochondrial apoptosis (39). Upon cleavage by caspase-8, BID generates the truncated form (tBID), which translocate to the mitochondria and interacts with BAX and BAK (40). The interaction of tBID with BAX and BAK induces their conformational changes and activation, promoting MOMP and the release of apoptotic factors (41).

p53 upregulated modulator of apoptosis (PUMA)

PUMA is a potent inducer of apoptosis and functions as a critical mediator of p53-dependent and -independent apoptotic pathways (42). Its transcriptional activation occurs in response to various cellular stresses, including DNA damage, oncogenic signalling, and hypoxia (43).

Bcl-2-associated death promoter (BAD)

In its unphosphorylated state, BAD forms heterodimers with BCL-2 or BCL-XL, preventing their interactions with pro-apoptotic effectors, BAX and BAK (44). Phosphorylation of specific serine residues on BAD promotes its dissociation from BCL-2 or BCL-XL, enabling the activation of BAX and BAK (45).

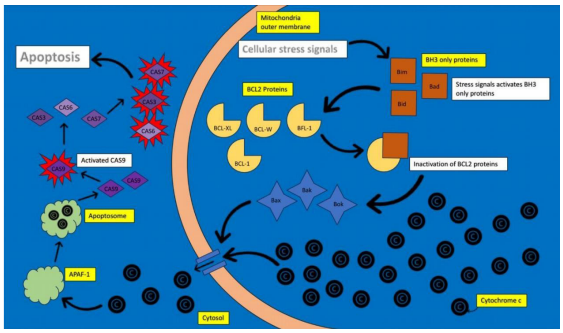

Figure 2: Intrinsic apoptotic pathway.

Table 1: BCL2 family members and their key regulators.

BCL Family Member	Structural Domains	Function	Cellular Localization	Interacting Proteins	Key Regulators
BCL-2	BH1, BH2, BH3, BH4	Anti-apoptotic	Mitochondrial membrane	BAX, BAK, BAD, BIM	BH3-only proteins, BH3 mimetics, BCL-2 homology 3 (BH3) peptides
BCL-XL	BH1, BH2, BH3, BH4	Anti-apoptotic	Mitochondrial membrane	BAX, BAK, BAD, BIM	BH3-only proteins, BH3 mimetics, BCL-2 homology 3 (BH3) peptides

MCL-1	BH1, BH2, BH, BH4	Anti-apoptotic	Mitochondrial membrane	BIM, NOXA	BH3-only proteins, BH3 mimetics, Mule, Mule-induced degradation
BCL-W	BH1, BH2, BH3, BH4	Anti-apoptotic	Mitochondria	BAX, BAK, BIM, BAD, PUMA	BH3-only proteins, BH3 mimetics (context-dependent)
BFL-1	BH1, BH3	Anti-apoptotic	Mitochondria	BAX, BAK, BIM, BAD, PUMA	BH3-only proteins, BH3 mimetics
BAX	BH1, BH2, BH3	Pro-apoptotic	Cytoplasm, Mitochondria	BCL-2, BCL-XL, MCL-1, BFL-1	BH3-only proteins, BH3 mimetics, tBID, PUMA, BIM, BAD, Bmf, Hrk, ART
BAK	BH1, BH2, BH3	Pro-apoptotic	Mitochondria	BCL-2, BCL-XL, MCL-1, BFL-1	BH3-only proteins, BH3 mimetics, tBID, PUMA, BIM, BAD, Bmf, Hrk, ART
BOK	BH1, BH2, BH3	Pro-apoptotic	Mitochondria	BCL-2, BCL-XL, MCL-1, BFL-1	BH3-only proteins, BH3 mimetics, tBID, PUMA, BIM, BAD, Bmf, Hrk, ART
BIM	BH3	BH3- domain only proteins.	Cytoplasm, Mitochondria	BCL-2, BCL-XL, MCL-1, BFL-1	JNK, p38, FOXO, ERK, Chk1
BID	BH3	BH3- domain only proteins.	Cytoplasm	BCL-2, BCL-XL, MCL-1, BCL-1	Caspase-8, caspase-3
PUMA	BH3	BH3- domain only proteins.	Cytoplasm, Mitochondria	BCL-2, BCL-XL, MCL-1, BFL-1	p53, p73, NOXA, BAX, BAK, BAD, MCL-1, BCL-XL, BCL-2
BAD	BH3	BH3- domain only proteins.	Cytoplasm	BCL-2, BCL-XL, MCL-1, BFL-1	Akt, PKA, Raf-1, ERK, JNK, Pim-1, B-Raf, PAK1, PKC, S6K, RSK, PDK1

BCL-2 and lung

Several studies have investigated the relationship between BCL-2 and lung cancer. In high-grade neuroendocrine lung cancers, BCL-2 has been identified as an acquired vulnerability and a potential therapeutic target (46). Targeted inhibition of BCL2 has shown efficacy in overcoming resistance to chemotherapy in non-small cell lung cancer (NSCLC) cell lines (47). However, the prognostic importance of BCL-2 expression in lung cancer is still debated. While some studies have shown that BCL-2-negative expression is associated with poor prognosis[47], a systematic review of studies in non-small cell lung cancer revealed conflicting results, with smaller studies showing a significant relationship between BCL-2 expression and risk of dying, while larger studies showed non-significant effects (48).

The regulation of BCL-2 expression and its impact on lung cancer progression have also been investigated. For example, nicotine

has been found to induce BCL-2 phosphorylation, leading to increased survival of lung cancer cells (49). Additionally, the transcription factor Runt-related transcription factor 2 (RUNX2) has been implicated in inhibiting the apoptosis process in lung cancer, and its knockdown has been shown to downregulate the expression of BCL-2 (50). Furthermore, the interaction between BCL-2 and other molecules has been explored. BCL-2-associated athanogene 3 (BAG3), a member of the BAG family, has been found to have a tight relationship with BCL-2 and can synergistically act with BCL-2 to induce anti-apoptotic effects in lung cancer (51). The miR-497/BCl-2 axis has also been identified as a potential therapeutic target in lung cancer, as miR-497 can decrease resistance to cisplatin by targeting BCL-2 (52).

BCL-2 plays a complex role in lung cancer, with both therapeutic and prognostic implications. Targeted inhibition of BCL-2 has shown promise in overcoming resistance to chemotherapy in NSCLC, while the prognostic significance of BCL-2 expression in lung cancer remains controversial. Further research is needed to fully understand the mechanisms underlying the relationship between BCL-2 and lung cancer and to explore its potential as a therapeutic target.

BCL-2 and breast cancer

Early studies have shown that BCL-2 expression is associated with low-grade, slowly proliferating Oestrogen positive (ER+) breast tumours, and its correlation with ER status is attributed to the improved survival observed in these tumours[53]. Recent studies have further supported the clinical validity of BCL2 as a prognostic marker for early-stage breast cancer, independent of ER, Human Epidermal Growth Factor Receptor 2 (HER2), and adjuvant therapy received (53). BCL-2 expression has been associated with favourable 5-year recurrence-free survival (RFS) and disease-specific survival (DSS) in luminal A breast cancer[54]. However, the prognostic role of BCL-2 expression in breast cancer is subtype-specific, and its significance in other subtypes remains unclear (54). In addition to its prognostic value, BCL-2 has been investigated in relation to other factors in breast cancer. High BCL-2 protein expression has been associated with a favourable outcome regardless of ER, Progesterone (PR), or HER2 status (55). On the other hand, BCL2 expression is only observed in a small proportion of triple-negative breast cancers (55). Furthermore, BCL-2 has been studied in the context of genetic polymorphisms. A study found that the BCL-2 C (-938) A gene polymorphism was associated with an increased risk of developing breast cancer (56). However, another study did not find an association between a BCL-2 promoter polymorphism (rs2279115) and BCL-2 expression or overall survival in breast cancer patients (57). The relationship between BCL-2 and breast cancer has also been explored in terms of its interaction with other molecules. miR-181a-5p has been found to downregulate BCL-2, leading to apoptosis in breast cancer cells (58). Additionally, BCL-2 expression has been correlated with p52 expression in breast carcinoma, suggesting a potential relationship between the two (59).

Breast cancer research has focused on BCL-2, whose expression has been linked to a variety of clinicopathologic traits and prognoses. When it comes to specific breast cancer subtypes, particularly luminal A tumours, BCL-2 has demonstrated prognostic relevance. Research is still being done on its importance in various subtypes of breast cancer as well as how it affects prognosis. Furthermore, studies have shown BCL-2 interacts with several other elements, including genetic polymorphisms and molecular interactions. To completely comprehend BCL-2's function in breast cancer and its potential as a therapeutic target, more research is required.

BCL-2 and prostrate cancer

Overexpression of BCL-2 has been associated with adverse prognostic factors,

disease progression, and therapy resistance in prostate cancer (60). High BCL-2 expression has been correlated with higher Gleason scores and lower biochemical recurrence-free survival in patients with advanced prostate cancer (61). Additionally, BCL-2 has been implicated in the development of castration-resistant prostate cancer (62). The regulation of BCL-2 in prostate cancer has also been investigated. The Mouse Double Minute 2 homolog (MDM2) oncogene, which has ubiquitin ligase activity, may have a direct role in BCL-2 regulation (63). The transcription factor RUNX2 has been shown to bind to the promoter region of antiapoptotic genes, including BCL-2, in prostate cancer cells (50). Furthermore, microRNA-205 has been identified as a regulator of BCL-2 in prostate cancer, with repression of BCL-2 by miR-205 being confirmed through reporter assays and western blotting (64). The relationship between BCL-2 and other factors in prostate cancer has also been explored. Down-regulation of BCL-2 has been associated with self-reported fatigue in non-metastatic prostate cancer patients receiving external beam radiation therapy (65). Additionally, a study found that a BCL-2 polymorphism (-938 C>A) showed a protective role in susceptibility to papillary thyroid carcinoma (66). BCL-2 has been studied in relation to prostate cancer, and its overexpression has been associated with adverse prognostic factors, disease progression, and therapy resistance. The regulation of BCL-2 in prostate cancer involves various mechanisms, including the involvement of the MDM2 oncogene, the transcription factor RUNX2, and microRNA-205. Further research is needed to fully understand the role of BCL-2 in prostate cancer and its potential as a therapeutic target.

BCL-2 as therapeutic targets to treat cancer

There are several types of therapeutic inhibitors that target BCL-2 in various cancers. These inhibitors include microtubule-directed agents, protein phosphatase 1/2A inhibitors, bromodomain and extra-terminal (BET) protein inhibitors, autophagy inhibitors, mitochondrial respiration inhibitors, ceramide metabolism inhibitors, epigenetic therapy, and MDM2 inhibitors. Microtubule-directed agents, such as taxol and nocodazole, have been shown to induce BCL-2 phosphorylation (67). Protein phosphatase 1/2A inhibitors, such as okadaic acid, can also induce BCL-2 phosphorylation (67). These agents target BCL-2 through different pathways, including extracellular signal-regulated kinase activation and G2/M accumulation (67). BET protein inhibitors have been investigated for their potential to increase sensitivity to BCL-2 inhibitors in chronic lymphocytic leukaemia (CLL) (68). These inhibitors work by inhibiting bromodomain and extra-terminal proteins, which can enhance the effectiveness of BCL-2 inhibitors like venetoclax (68). Autophagy inhibitors have also been explored as therapeutic agents targeting BCL-2. Inhibition of the dissociation of the Beclin1 and BCL-2 complex, a negative regulator of autophagy, has been shown to be a potential strategy for developing autophagy inhibitors (69). These inhibitors can be useful in the treatment of diseases involving autophagy dysregulation, including cancer and viral infections (69). Mitochondrial respiration inhibitors have been investigated for their potential to target BCL-2 in high-grade MYC-associated B-cell lymphoma (70). These inhibitors can disrupt mitochondrial metabolism and induce cancer cell death, potentially synergizing with BCL-2 inhibitors (70). Ceramide metabolism inhibitors have also been explored as potential therapeutics targeting BCL-2. Inhibition of ceramide metabolism can sensitize leukaemia cells to inhibition of BCL-2 like proteins, leading to enhanced apoptosis (71). Epigenetic therapy has been shown to activate endogenous retroelements, which remodel mitochondrial metabolism and sensitize cancer cells to BCL-2 inhibitors (72). This combination therapy has shown efficacy in acute myeloid leukaemia and may have potential for other cancers (72). MDM2 inhibitors, such as nutlin-3a, have been investigated for their ability to activate the p53 pathway and overcome BCL-2 overexpression in lymphoma. Combination therapy of MDM2 inhibitors with

BCL-2 inhibitors has shown synergistic effects in inducing apoptosis in leukaemia (73).

Conclusion

Apoptosis and programmed cell death are tightly regulated by the BCL-2 protein family. The family includes both proapoptotic and antiapoptotic members that, in turn, either encourage or prevent the release of cytochrome c from mitochondria. Through the creation of pores in the outer mitochondrial membrane, the proapoptotic proteins BAX and BAK directly mediate the release of cytochrome c. The antiapoptotic members, on the other hand, like BCL-2 and BCL-XL, stop the release of cytochrome c and support cell survival. BH3-only proteins are a diverse group of proteins that regulate apoptosis and autophagy. They can be classified into BH3-only proteins, sensitizers, and activators. These proteins play a critical role in promoting apoptosis by activating proapoptotic effectors or neutralizing antiapoptotic proteins. BH3-only proteins also contribute to the induction of autophagy. Understanding the structural and functional aspects of BH3-only proteins is important for developing targeted therapies for cancer and other diseases.

References

1. Kehr, S. and Vogler, M., 2021. It's time to die: BH3 mimetics in solid tumors. *Biochimica et Biophysica Acta (BBA)-Molecular Cell Research*, *1868*(5), p.118987.

2. Lernoux, M., Schnekenburger, M., Dicato, M. and Diederich, M., 2021. Susceptibility of multiple myeloma to B-cell lymphoma 2 family inhibitors. *Biochemical Pharmacology*, *188*, p.114526.

3. Green, D.R., 2022. The mitochondrial pathway of apoptosis part II: the BCL-2 protein family. *Cold Spring Harbor perspectives in biology*, *14*(6), p.a041046.

4. Kaloni, D., Diepstraten, S.T., Strasser, A. and Kelly, G.L., 2023. BCL-2 protein family: Attractive targets for cancer therapy. *Apoptosis*, *28*(1), pp.20-38.

5. Di Fusco, D., Di Grazia, A., Di Maggio, G., Segreto, M.T., Iannucci, A., Maresca, C., De Stefano, A., Sica, G., Stolfi, C., Monteleone, G. and Monteleone, I., 2023. A novel tumour enhancer function of Insulin-like growth factor II mRNA-binding protein 3 in colorectal cancer. *Cell Death & Disease*, *14*(4), p.243.

6. Fitzgerald, M.C., O'Halloran, P.J., Connolly, N.M. and Murphy, B.M., 2022. Targeting the apoptosis pathway to treat tumours of the paediatric nervous system. *Cell Death & Disease*, *13*(5), p.460.

7. Azarbarzin, S., Hosseinpour-Feizi, M.A., Khojasteh, S.M.B., Baradaran, B. and Safaralizadeh, R., 2021. MicroRNA-383-5p restrains the proliferation and migration of breast cancer cells and promotes apoptosis via inhibition of PD-L1. *Life sciences*, *267*, p.118939.

8. Abd-Allah, G.M., Ismail, A., El-Mahdy, H.A., Elsakka, E.G., El-Husseiny, A.A., Abdelmaksoud, N.M., Salman, A., Elkhawaga, S.Y. and Doghish, A.S., 2023. miRNAs as potential game-changers in melanoma: a comprehensive review. *Pathology-Research and Practice*, p.154424.

9. Yi, J., Kellner, V., Joo, H., Chien, N., Patel, S., Chaban, Z. and Tsai, J., 2023. Characterizing the consensus residue specificity and surface of BCL-2 binding to BH3 ligands using the Knob-Socket model. *PloS one*, *18*(2), p.e0281463.

10. Calis, S., Dogan, B., Durdagi, S., Celebi, A., Yapicier, O., Kilic, T., Turanli, E.T. and Avsar, T., 2022. A novel BH3 mimetic Bcl-2 inhibitor promotes autophagic cell death and reduces in vivo Glioblastoma tumor growth. Cell Death Discovery, 8(1), p.433.

11. Ngoi, N.Y.L., Choong, C., Lee, J., Bellot, G., Wong, A.L., Goh, B.C. and Pervaiz, S., 2020. Targeting mitochondrial apoptosis to overcome treatment resistance in cancer. *Cancers*, *12*(3), p.574.

12. Zhang, Z., Bai, L., Hou, L., Deng, H., Luan, S., Liu, D., Huang, M. and Zhao, L., 2022. Trends in targeting Bcl-2 anti-apoptotic proteins for cancer treatment. *European Journal of Medicinal Chemistry*, *232*, p.114184.

13. Wu, R.H., Zhu, C.Y., Yu, P.H., Ma, Y., Hussain, L., Naranmandura, H. and Wang, Q.Q., 2023. The landscape of novel strategies for acute myeloid leukemia treatment: Therapeutic trends, challenges, and future directions. *Toxicology and Applied Pharmacology*, *473*, p.116585.

14. Arulananda, S., Lee, E.F., Fairlie, W.D. and John, T., 2021. The role of BCL-2 family proteins and therapeutic potential of BH3-mimetics in malignant pleural mesothelioma. *Expert review of anticancer therapy*, *21*(4), pp.413-424.

15. Knight, T., Luedtke, D., Edwards, H., Taub, J.W. and Ge, Y., 2019. A delicate balance–The BCL-2 family and its role in apoptosis, oncogenesis, and cancer therapeutics. *Biochemical pharmacology*, *162*, pp.250-261.

16. Vandenabeele, P., Bultynck, G. and Savvides, S.N., 2023. Pore-forming proteins as drivers of membrane permeabilization in cell death pathways. *Nature Reviews Molecular Cell Biology*, *24*(5), pp.312-333.

17. Jang, D.M., Oh, E.K., Hahn, H., Kim, H.S. and Han, B.W., 2022. Structural insights into apoptotic regulation of human Bfk as a novel Bcl-2 family member. *Computational and Structural Biotechnology Journal*, *20*, pp.745-756.

18. Sharma, V.K., Singh, T.G., Singh, S., Garg, N. and Dhiman, S., 2021. Apoptotic pathways and Alzheimer's disease: probing therapeutic potential. *Neurochemical research*, *46*(12), pp.3103-3122.

19. Chota, A., George, B.P. and Abrahamse, H., 2021. Interactions of multidomain pro-apoptotic and anti-apoptotic proteins in cancer cell death. *Oncotarget*, *12*(16), p.1615.

20. Li, M., Wang, D., He, J., Chen, L. and Li, H., 2020. Bcl-XL: A multifunctional anti-apoptotic protein. *Pharmacological research*, *151*, p.104547.

21. Borrás, C., Mas-Bargues, C., Román-Domínguez, A., Sanz-Ros, J., Gimeno-Mallench, L., Inglés, M., Gambini, J. and Viña, J., 2020. BCL-xL, a mitochondrial protein involved in successful aging: from C. elegans to human centenarians. *International journal of molecular sciences*, *21*(2), p.418.

22. Huang, K., O'Neill, K.L., Li, J., Zhou, W., Han, N., Pang, X., Wu, W., Struble, L., Borgstahl, G., Liu, Z. and Zhang, L., 2019. BH3-only proteins target BCL-xL/MCL-1, not BAX/BAK, to initiate apoptosis. *Cell research*, *29*(11), pp.942-952.

23. Roufayel, R., Younes, K., Al-Sabi, A. and Murshid, N., 2022. BH3-only proteins Noxa and Puma are key regulators of induced apoptosis. *Life*, *12*(2), p.256.

24. Pan, P., Ge, W., Lei, Z., Luo, W., Liu, Y., Guan, Z., Chen, L., Yu, Z., Shen, M., Hu, D. and Xiang, Q., 2023. SARS-CoV-2 N protein enhances the anti-apoptotic activity of MCL-1 to promote viral replication. *Signal Transduction and Targeted Therapy*, *8*(1), p.194.

25. Khan, S., Basu, S., Raj, D. and Lahiri, A., 2023. Role of mitochondria in regulating

immune response during bacterial infection. In *International Review of Cell and Molecular Biology* (Vol. 374, pp. 159-200). Academic Press.

26. Anantram, A. and Degani, M., 2019. Targeting cancer's Achilles' heel: role of BCL-2 inhibitors in cellular senescence and apoptosis. *Future medicinal chemistry*, *11*(17), pp.2287-2312.

27. Yang, Y., Li, S., Wang, Y., Zhao, Y. and Li, Q., 2022. Protein tyrosine kinase inhibitor resistance in malignant tumors: molecular mechanisms and future perspective. *Signal transduction and targeted therapy*, *7*(1), p.329.

28. Klanova, M. and Klener, P., 2020. BCL-2 proteins in pathogenesis and therapy of B-cell non-Hodgkin lymphomas. *Cancers*, *12*(4), p.938.

29. Aksoy, O., Lind, J., Sunder-Plaßmann, V., Vallet, S. and Podar, K., 2023. Bone marrow microenvironment-induced regulation of Bcl-2 family members in multiple myeloma (MM): therapeutic implications. *Cytokine*, *161*, p.156062.

30. Delgado, Y., Torres, A. and Milián, M., 2019. Apoptosis' activation associated to BH3 only domain and BCL-2 homology domain proteins: new way to design anti-cancer drugs. *J Cancer Prev Curr Res*, *10*(3), pp.54-59.

31. Bock, F.J. and Tait, S.W., 2020. Mitochondria as multifaceted regulators of cell death. *Nature reviews Molecular cell biology*, *21*(2), pp.85-100.

32. Hollville, E., Romero, S.E. and Deshmukh, M., 2019. Apoptotic cell death regulation in neurons. *The FEBS journal*, *286*(17), pp.3276-3298.

33. González□Arzola, K., Guerra□Castellano, A., Rivero□Rodríguez, F., Casado□Combreras, M.Á., Pérez□Mejías, G., Díaz□Quintana, A., Díaz□Moreno, I. and De la Rosa, M.A., 2021. Mitochondrial cytochrome c shot towards histone chaperone condensates in the nucleus. *FEBS Open Bio*, *11*(9), pp.2418-2440.

34. Qian, S., Wei, Z., Yang, W., Huang, J., Yang, Y. and Wang, J., 2022. The role of BCL-2 family proteins in regulating apoptosis and cancer therapy. *Frontiers in oncology*, *12*, p.985363.

35. Kosuru, R., Vijayakumar, M.R. and Singh, S., 2021. Preclinical evidence of the therapeutic role of pterostilbene: Focus on the mitochondria. In *Mitochondrial Physiology and Vegetal Molecules* (pp. 301-315). Academic Press.

36. Flores□Romero, H., Hohorst, L., John, M., Albert, M.C., King, L.E., Beckmann, L., Szabo, T., Hertlein, V., Luo, X., Villunger, A. and Frenzel, L.P., 2022. BCL□2□family protein tBID can act as a BAX□like effector of apoptosis. *The EMBO journal*, *41*(2), p.e108690.

37. Stöhr, D., Jeltsch, A. and Rehm, M., 2020. TRAIL receptor signaling: From the basics of canonical signal transduction toward its entanglement with ER stress and the unfolded protein response. *International review of cell and molecular biology*, *351*, pp.57-99.

38. Kulbay, M., Paimboeuf, A., Ozdemir, D. and Bernier, J., 2022. Review of cancer cell resistance mechanisms to apoptosis and actual targeted therapies. *Journal of Cellular Biochemistry*, *123*(11), pp.1736-1761.

39. Tsuchiya, K., 2020. Inflammasome□associated cell death: pyroptosis, apoptosis, and physiological implications. *Microbiology and immunology*, *64*(4), pp.252-269.

40. Rogers, C., Erkes, D.A., Nardone, A., Aplin, A.E., Fernandes-Alnemri, T. and Alnemri, E.S., 2019. Gasdermin pores permeabilize mitochondria to augment caspase-3 activation during apoptosis and inflammasome activation. *Nature communications*, *10*(1), p.1689.

41. Mandal, R., Barrón, J.C., Kostova, I., Becker, S. and Strebhardt, K., 2020. Caspase-8: The double-edged sword. *Biochimica et Biophysica Acta (BBA)-Reviews on Cancer*, *1873*(2), p.188357.

42. Taghadosi, M., Adib, M., Jamshidi, A., Mahmoudi, M. and Farhadi, E., 2021. The p53 status in rheumatoid arthritis with focus on fibroblast-like synoviocytes. *Immunologic research*, *69*(3), pp.225-238.

43. Liu, Y., Leslie, P.L. and Zhang, Y., 2021. Life and death decision-making by p53 and implications for cancer immunotherapy. *Trends in cancer*, *7*(3), pp.226-239.

44. Wolf, E., Lento, C., Pu, J., Dickinson, B.C. and Wilson, D.J., 2023. Innate Conformational Dynamics Drive Binding Specificity in Anti-Apoptotic Proteins Mcl-1 and Bcl-2. *Biochemistry*, *62*(11), pp.1619-1630.

45. Bernhem, K., Fontana, J.M., Svensson, D., Zhang, L., Nilsson, L.M., Scott, L., Blom, H., Brismar, H. and Aperia, A., 2021. Super-resolution microscopy reveals that Na+/K+-ATPase signaling protects against glucose-induced apoptosis by deactivating Bad. *Cell Death & Disease*, *12*(8), p.739.

46. Augustyn, A., Borromeo, M., Wang, T., Fujimoto, J., Shao, C., Dospoy, P.D., Lee, V., Tan, C., Sullivan, J.P., Larsen, J.E. and Girard, L., 2014. ASCL1 is a lineage oncogene providing therapeutic targets for high-grade neuroendocrine lung cancers. *Proceedings of the National Academy of Sciences*, *111*(41), pp.14788-14793.

47. Lu, C., Chen, H., Shan, Z. and Yang, L., 2016. Identification of differentially expressed genes between lung adenocarcinoma and lung squamous cell carcinoma by gene expression profiling. *Molecular Medicine Reports*, *14*(2), pp.1483-1490.

48. Altman, D.G., McShane, L.M., Sauerbrei, W. and Taube, S.E., 2012. Reporting recommendations for tumor marker prognostic studies (REMARK): explanation and elaboration. *BMC medicine*, *10*, pp.1-39.

49. Xin, M. and Deng, X., 2005. Nicotine inactivation of the proapoptotic function of Bax through phosphorylation. *Journal of Biological Chemistry*, *280*(11), pp.10781-10789.

50. Bernal, C., Otalora, A., Cañas, A., Barreto, A., Prieto, K., Montecino, M. and Rojas, A., 2022. Regulatory role of the RUNX2 transcription factor in lung cancer apoptosis. *International Journal of Cell Biology*, *2022*(1), p.5198203.

51. Sun, H., Liu, K., Huang, J., Sun, Q., Shao, C., Luo, J., Xu, L., Shen, Y. and Ren, B., 2019. FAM111B, a direct target of p53, promotes the malignant process of lung adenocarcinoma. *OncoTargets and therapy*, pp.2829-2842.

52. Kattan, S.W., Hobani, Y.H., Abubakr Babteen, N., Alghamdi, S.A., Toraih, E.A., Ibrahiem, A.T., Fawzy, M.S. and Faisal, S., 2022. Association of B-cell lymphoma 2/microRNA-497 gene expression ratio score with metastasis in patients with colorectal cancer: A propensity-matched cohort analysis. *Journal of clinical laboratory analysis*, *36*(2), p.e24227.

53. Hwang, K.T., Woo, J.W., Shin, H.C., Kim, H.S., Ahn, S.K., Moon, H.G., Han, W., Park, I.A. and Noh, D.Y., 2012. Prognostic influence of BCL2 expression in breast cancer. *International journal of cancer*,

131(7), pp.E1109-E1119.

54. Eom, Y.H., Kim, H.S., Lee, A., Song, B.J. and Chae, B.J., 2016. BCL2 as a subtype-specific prognostic marker for breast cancer. *Journal of breast cancer*, *19*(3), p.252.

55. Campbell, K.J., Dhayade, S., Ferrari, N., Sims, A.H., Johnson, E., Mason, S.M., Dickson, A., Ryan, K.M., Kalna, G., Edwards, J. and Tait, S.W., 2018. MCL-1 is a prognostic indicator and drug target in breast cancer. *Cell death & disease*, *9*(2), p.19.

56. Ozoran, E., Trabulus, F.D.C., Erhan, D., Batar, B. and Guven, M., 2022. Association of XRCC3, XRCC4, BAX, and BCL-2 Polymorphisms with the Risk of Breast Cancer. *International Journal of Breast Cancer*, *2022*(1), p.5817841.

57. Searle, C.J., Brock, I.W., Cross, S.S., Balasubramanian, S.P., Reed, M.W. and Cox, A., 2012. A BCL2 promoter polymorphism rs2279115 is not associated with BCL2 protein expression or patient survival in breast cancer patients. *Springerplus*, *1*, pp.1-8.

58. Xu, G., Yang, Y., Yang, J., Xiao, L., Wang, X., Qin, L., Gao, J., Xuan, R., Wu, X., Chen, Z. and Sun, R., 2023. Screening and identification of miR-181a-5p in oral squamous cell carcinoma and functional verification in vivo and in vitro. *BMC cancer*, *23*(1), p.162.

59. Al-Sudani, I.M., AlKhafaji, A.H.M.A. and Mohammed, R.S., 2023. Correlation Expression between P52 and BCL2 among Iraqi Women with Breast Carcinoma. *Baghdad Science Journal*, *20*(2), pp.0278-0278.

60. Pilling, A.B. and Hwang, C., 2019. Targeting prosurvival BCL2 signaling through Akt blockade sensitizes castration-resistant prostate cancer cells to enzalutamide. *The Prostate*, *79*(11), pp.1347-1359.

61. Renner, W., Langsenlehner, U., Krenn-Pilko, S., Eder, P. and Langsenlehner, T., 2017. BCL2-Genotypen und Überleben bei Prostatakrebs. *Strahlentherapie und Onkologie*, *193*, pp.466-471.

62. Mitsui, Y., Chang, I., Kato, T., Hashimoto, Y., Yamamura, S., Fukuhara, S., Wong, D.K., Shiina, M., Imai-Sumida, M., Majid, S. and Saini, S., 2016. Functional role and tobacco smoking effects on methylation of CYP1A1 gene in prostate cancer. *Oncotarget*, *7*(31), p.49107.

63. Zhang, Z., Li, M., Wang, H., Agrawal, S. and Zhang, R., 2003. Antisense therapy targeting MDM2 oncogene in prostate cancer: Effects on proliferation, apoptosis, multiple gene expression, and chemotherapy. *Proceedings of the National Academy of Sciences*, *100*(20), pp.11636-11641.

64. Verdoodt, B., Neid, M., Vogt, M., Kuhn, V., Liffers, S.T., Palisaar, R.J., Noldus, J., Tannapfel, A. and Mirmohammadsadegh, A., 2013. MicroRNA-205, a novel regulator of the anti-apoptotic protein Bcl2, is downregulated in prostate cancer. *International journal of oncology*, *43*(1), pp.307-314.

65. Hsiao, C.P., Wang, D., Kaushal, A. and Saligan, L., 2013. Mitochondria-related gene expression changes are associated with fatigue in patients with nonmetastatic prostate cancer receiving external beam radiation therapy. *Cancer nursing*, *36*(3), pp.189-197.

66. Li, X., He, J., Zhou, M., Cao, Y., Jin, Y. and Zou, Q., 2019. Identification and validation of core genes involved in the development of papillary thyroid carcinoma via bioinformatics analysis. *International Journal of Genomics*, *2019*(1), p.5894926.

67. Domina, A.M., Smith, J.H. and Craig, R.W., 2000. Myeloid cell leukemia 1 is phosphorylated through two distinct pathways, one associated with extracellular signal-regulated kinase activation and the other with G2/M accumulation or protein phosphatase 1/2A inhibition. *Journal of Biological Chemistry*, *275*(28), pp.21688-21694.

68. Carrà, G., Nicoli, P., Lingua, M.F., Maffeo, B., Cartellà, A., Circosta, P., Brancaccio, M., Parvis, G., Gaidano, V., Guerrasio, A. and Saglio, G., 2020. Inhibition of bromodomain and extra-terminal proteins increases sensitivity to venetoclax in chronic lymphocytic leukaemia. *Journal of Cellular and Molecular Medicine*, *24*(2), pp.1650-1657.

69. Dai, J.P., Zhao, X.F., Zeng, J., Wan, Q.Y., Yang, J.C., Li, W.Z., Chen, X.X., Wang, G.F. and Li, K.S., 2013. Drug screening for autophagy inhibitors based on the dissociation of Beclin1-Bcl2 complex using BiFC technique and mechanism of eugenol on anti-influenza A virus activity. *PloS one*, *8*(4), p.e61026.

70. Donati, G., Ravà, M., Filipuzzi, M., Nicoli, P., Cassina, L., Verrecchia, A., Doni, M., Rodighiero, S., Parodi, F., Boletta, A. and Vellano, C.P., 2022. Targeting mitochondrial respiration and the BCL2 family in high-grade MYC-associated B-cell lymphoma. *Molecular oncology*, *16*(5), pp.1132-1152.

71. Casson, L., Howell, L., Mathews, L.A., Ferrer, M., Southall, N., Guha, R., Keller, J.M., Thomas, C., Siskind, L.J. and Beverly, L.J., 2013. Inhibition of ceramide metabolism sensitizes human leukemia cells to inhibition of BCL2-like proteins. *PloS one*, *8*(1), p.e54525.

72. Fresquet, V., Garcia-Barchino, M.J., Larrayoz, M., Celay, J., Vicente, C., Fernandez-Galilea, M., Larrayoz, M.J., Calasanz, M.J., Panizo, C., Junza, A. and Han, J., 2021. Endogenous retroelement activation by epigenetic therapy reverses the Warburg effect and elicits mitochondrial-mediated cancer cell death. *Cancer Discovery*, *11*(5), pp.1268-1285.

73. Drakos, E., Singh, R.R., Rassidakis, G.Z., Schlette, E., Li, J., Claret, F.X., Ford, R.J., Vega, F. and Medeiros, L.J., 2011. Activation of the p53 pathway by the MDM2 inhibitor nutlin-3a overcomes BCL2 overexpression in a preclinical model of diffuse large B-cell lymphoma associated with t (14; 18)(q32; q21). *Leukemia*, *25*(5), pp.856-867.