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Research Article

Determination of cytotoxicity of selected plant extracts and flavonoids using brine shrimp assay

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Abstract

Cytotoxic potential of selected plant extracts and flavonoids was determined using brine shrimp assay. The plant extracts selected were tulasi, spinach and ajwain. The flavonoids/polyphenols selected were kaempferol and eugenol. To determine cytotoxicity, *Artemia salina* (brine shrimp) were hatched using artificial seawater (37 g NaCl/L). Then, the brine shrimp were incubated in plant extracts with concentrations of 100, 30, 10, 3 and 1 µg/mL. Similarly, different concentrations of flavonoids at 100, 30, 10, 3 and 1 µg/mL were taken to incubate brine shrimp for a period of 24 h. After 24 h, number of dead and live nauplii were counted. The data obtained was analysed to get the LD_{50} values of the plant extracts and flavonoids. Results showed that the plant extract ajwain was least potent against brine shrimp with obtained LD_{50} values of 5.01 and 7.52 µg/mL from graphical and Karber's methods, respectively, and eugenol was most potent with an LD_{50} value of 1.6 µg/mL. Therefore, the obtained results support that the selected plant extracts and flavonoids were bioactive compounds.

Keywords: Brine shrimp, Cytotoxicity, Flavonoids, $LD_{50,}$ Plant extracts

Highlights

- Of all the plant extracts, tulasi plant extract has the highest cytotoxic potential.
- The flavonoid eugenol present in tulasi has the highest cytotoxic potential compared to the flavonoid kaempferol present in spinach.
- Flavonoid has shown more potency than the whole plant extract.

INTRODUCTION

In recent times, the inclination towards therapy with natural products has increased due to the advantage of minimal adverse effects in treatment. Before using the phytochemicals for treatment, it is necessary to know their LD_{50} for the safe use of these compounds. Tulasi (*Ocimum sanctum*) is a well-known medicinal plant and is even known as an emporium of medicinal plants (Borah *et al*., 2018). Spinach (*Spinacia oleracea*) is a leafy vegetable which is rich in flavonoids and polyphenols (Montenegro *et al*., 2022). It is known to have protective effects on various organs of the body. Ajwain (*Trachyspermum ammi* L.) is known to have medicinal properties and help in various gastric disturbances (Boskabady *et al*., 2014). Flavonoids are a broad class of polyphenolic substances that are primarily present in fruits, vegetables, and medicinal plants (Babu *et al*., 2009). It is important that any agent has a bioactive component in order to use it for beneficial outcomes. So, it is necessary to screen them for their bioactivit/ cytotoxic potential. Brine shrimp is a simple zoological organism used to assess the cytotoxicity of natural compounds. It is known as *Artemia salina* and is used as a preliminary screening tool to evaluate the bioactivity of compounds (Parvez *et al*., 2016). This assay is reliable for toxicity studies and is used widely across the world. The viability of its larvae helps in assessing the toxic dose of a compound. It is simple to perform, rapid and inexpensive. This assay is being used to screen the toxicity of various plant extracts (Mc Laughlin *et al*., 1991), phytochemicals and heavy metals (Martinez *et al*., 1999). This assay acts as a preliminary screening tool for compounds that need to be screened for toxicity studies.

MATERIALS AND METHODS

Brine shrimp assay is easy to perform and is widely used to know the bioactivity of natural compounds. The eggs of *Artemia salina* can be easily procured from local aquarium shops. It is generally used as fish food. Brine shrimp eggs were purchased from a brand called PEQON from local shops selling aquarium and aqua culture-related material in Vijayawada. Brine shrimp

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eggs were being sold as fish food in a pet shop called 'Fluffy Pets' in Vijayawada, Andhra Pradesh. The eggs were stored at room temperature (24ºC) and were handled without touching with bare hands. The eggs need to be released directly into the water when opened.

Instruments and reagents: A conical tube is required for hatching the brine shrimp, and plastic vials are required for their incubation. An aerator for the supply of oxygen, a table lamp for the light source, a Pasteur pipette for handling brine shrimp and a magnifying glass for counting them are needed. Sodium chloride and distilled water are required to make seawater.

Hatching of brine shrimp: The procured brine shrimp (*Artemia salina*) eggs were hatched using artificial seawater. It was prepared by addition of 37 g of sodium chloride in 1L of distilled water. The prepared water was added to a cone-shaped bottom container. A teaspoon of *Artemia salina* eggs or a single capsule of eggs was added to the artificially prepared seawater. An aerator was arranged in the container to provide adequate aeration to the eggs. A light source was also arranged for the hatched nauplii to come to the surface as the larvae of brine shrimp show phototaxis. These nauplii that were generated from the hatching of eggs after 24-36 h were used to perform the cytotoxicity assay.

Preparation of control group: There are two control groups in the present study. In general, vincristine sulphate, podophyllum toxin, and potassium permanganate are used as control agents for brine shrimp cytotoxicity assays. In the present study, potassium permanganate ($KMnO_4$) is used as a positive control group in 5 different concentrations: 100, 30, 10, 3 and 1 µg/mL from a stock solution of 1mg/mL. The negative control group used was artificial sea water that was prepared earlier without any agent.

Preparation of plant extracts samples: The plant extracts used in the present study were tulasi, spinach and ajwain. Leaves of these plants were taken and were removed of dust and dirt. Then, they are dried and powdered. The extracts were prepared by using acetone as a solvent in 1:10 ratio by keeping the solvent mixture on an orbital shaker for 72 h. The extract was filtered from solvent by using Whatman filter paper-1. The filtrate was kept in a hot air oven at 50ºC till the filtrate was evaporated. The extract obtained was dissolved in acetone as a 100 mg/mL stock solution and stored in a refrigerator. The chosen extracts were subjected to phyto-chemical analysis as per the standard procedures for further study. Extracts of tulasi and spinach showed positive for flavonoids by lead acetate test.

For the assay, 1 mg/mL of stock solutions of tulasi, spinach and ajwain extracts were prepared in artificial seawater solution to achieve definite final concentrations. Five doses of each plant extract, 100, 30, 10, 3 and 1 µg/mL, were used for the cytotoxicity assay.

Preparation of flavonoid samples: The flavonoids used in the present study are eugenol and kaempferol. For the assay a 1 mg/mL of stock solutions of both eugenol and kaempferol were prepared in artificial seawater solution to achieve definite final concentrations. Five doses of each flavonoid, 100, 30, 10, 3 and 1 µg/mL were used for the cytotoxicity assay.

Addition of brine shrimp larvae: Five vials for each plant extract sample and 5 vials for each flavonoid at predetermined concentrations (100, 30, 10, 3 and 1 µg/mL) prepared in seawater were taken. Similarly, five vials containing $KMNO_4$ were also taken along with a vial that contained only seawater. Proper labelling of all the vials is mandatory. To the vials 10 nauplii were added using a Pasteur pipette with the help of a magnifying lens. The set up was kept in a dark room for 24 h.

Counting of brine shrimp larvae: After 24 h of incubation, the dead and live nauplii were counted with the help of a magnifying lens in both sample and control groups. The larvae were considered dead if they did not show any significant motility after a few seconds of observation. Each test was conducted five times to have statistically measurable data for analysis.

From the live and dead nauplii count, the mortality of the nauplii was calculated using the following formula:

$$\% \text{ Mortality} = \frac{\text{Total nauplii} - \text{Alive nauplii}}{\text{Total nauplii}} \text{ X } 100$$

Analysis of data: The dose and mortality percentage were analysed by using both arithmetic method (Karber's method) and graphical method (Ghosh, 2007).

RESULTS

The results of exposing *Artemia salina* to different concentrations showed that there was 100% mortality in the positive control ($KMnO_4$) group, as shown in

Fig. 1. There was no mortality in the negative control group, i.e. artificial seawater. The mortality percentage ranged from 0% to 100% in the different groups used. The LD_{50} values were determined by plotting % mortality on Y-axis and log dose on X-axis.

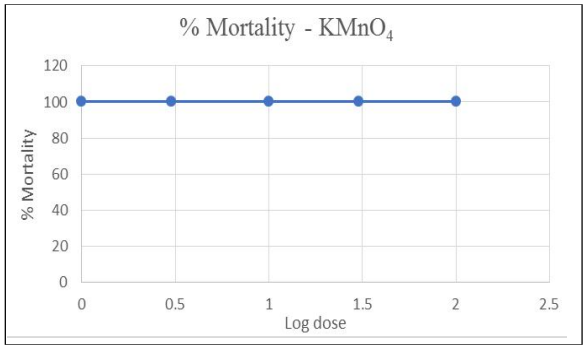


Fig. 1. Log dose concentration of $KMnO_4$ Vs % mortality of nauplii

The LD_{50} values were determined by using both graphical and Karber's methods. From the graphical method, the LD_{50} values of the plant extracts tulasi, spinach and ajwain were observed as 3.55, 4.47 and 5.01 µg/mL, respectively (Fig. 2). The LD_{50} values of the flavonoids eugenol and kaempferol by the graphical method were observed as 1 and 3.01 µg/mL, respectively (Fig. 3).

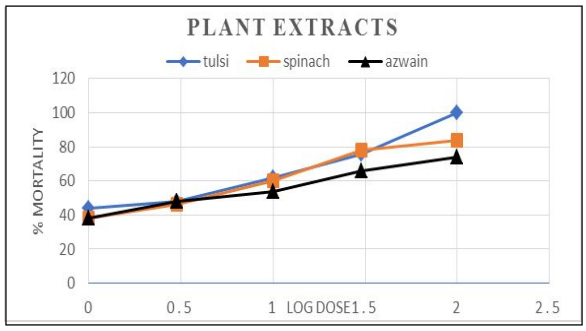


Fig. 2. Log dose concentration of tulasi, spinach and ajwain Vs % mortality of nauplii

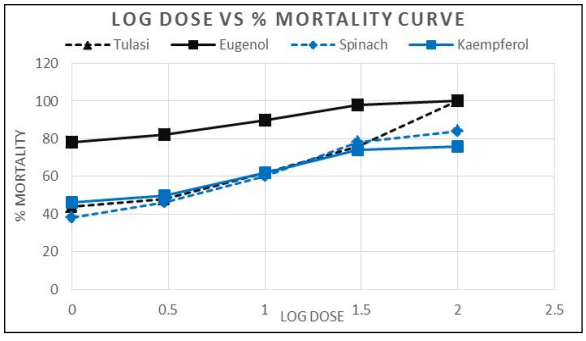


Fig. 3. Log dose concentration of tulasi and spinach with their respective flavonoid eugenol and kaempferol Vs % mortality of nauplii

From Karber's method, the LD_{50} values of the plant extracts were observed as 5.06, 5.89 and 7.52 µg/mL, respectively. The LD_{50} values for the flavonoids eugenol and kaempferol from Karber's method were observed as 1.6 and 5.78 µg/mL, respectively.

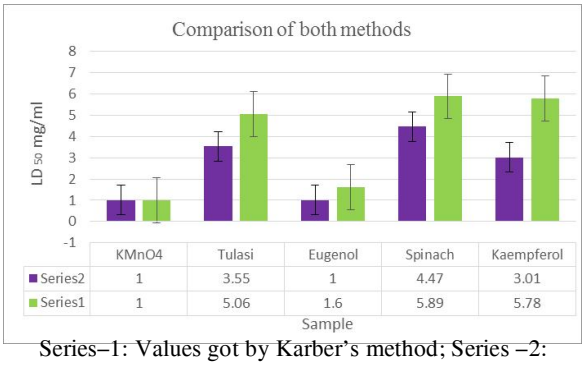
Both methods showed that the plant extract ajwain has a high LD_{50} value, which indicates it is the least potent. So, further cytotoxicity assay was carried out for the potent plant extracts by comparing them with the active flavonoid present in it. Eugenol was taken as the main flavonoid in tulasi (Prakash *et al*., 2007) and kaempferol in spinach (Dabeek *et al*., 2019).

DISCUSSION

The cytotoxicity assay on the plant extracts and flavonoids showed that all of them contained bioactive components. Their LD_{50} values represent the lethality of 50% of the brine shrimp nauplii due to the bioactive components present in the tulasi, spinach, ajwain, kaempferol and eugenol. The order of lethality of these components used was observed to be almost identical with minor differences. There is a dose dependent lethality of the nauplii in the plant extract and flavonoid groups. The highest mortality was observed at 100 µg/mL concentration.

- The order of lethality observed by Karber's method was as follows: Eugenol> Tulasi> Kaempferol> Spinach.
- The order of lethality observed by the graphical method was as follows: Eugenol> Kaempferol> Tulasi> Spinach.

Although some differences exist between both methods, it suggests that the flavonoid present in the particular plant extract is more potent than the whole plant extract itself. From the LD_{50} values, it is evident that eugenol is more potent than its plant extract tulasi. Similarly, kaempferol is more potent than its plant extract



Series–1: Values got by Karber's method; Series –2: Values got by Graphical method

Fig. 4. Representation of LD_{50} values of plant extracts and flavonoids by both methods

Table 1. Calculation of LD_{50}, regression equation and R^2 value

Sl. No.	Sample taken	LD_{50} (µg/mL) Graphical method	LD_{50} (µg/mL) Karber's method	Regression equation	R^2 value
1.	Tulasi plant extract	3.55	5.06	y = 12.189ln(x) + 38.191	0.95
2.	Spinach plant extract	4.47	5.89	y = 10.761ln(x) + 36.648	0.98
3.	Ajwain plant extract	5.01	7.52	y = 7.8074ln(x) + 38.187	0.99
4.	Eugenol (Flavonoid in tulasi)	<1	1.6	y = 5.207ln(x) + 77.72	0.97
5.	Kaempferol (Flavonoid in spinach)	3.01	5.78	y = 7.4696ln(x) + 44.158	0.95

spinach. R^2 values were included to represent how well the data fits with the experiment (Table 1, Fig. 4). A good correlation will be found if R^2 is close to 1.

Brine shrimp assay is a convenient method to test the cytotoxic potential of various natural compounds. Wu (2014) also mentioned in his article that Brine shrimp assay is best for high throughput screening of bioactive compounds. It is easy to perform, and the results are reliable for preliminary studies. However, more specific assays need to be performed to draw conclusions (Meyer *et al*., 1992). Similar studies were performed by Konan *et al.* (2022), where they used a brine shrimp lethality assay to screen the toxicity of *Imperata cylindrical* leaf extracts.

Based on the results obtained, it can be concluded that the bioactive components present in the whole plant extracts are more potent than the plant extract. Of all the selected plant extracts and flavonoids, eugenol is the most potent. Of the plant extracts, tulasi is the most potent.

Conflict of interest: Authors have no conflict of interest in the study.

Author's contribution: GSP, PA: Performed the study and statistical analysis; GS, GSR: Design of the study.

Data availability statement: All the data supporting the research findings have been presented in this paper. The corresponding author is willing to provide the raw data upon reasonable request.

REFERENCES

Babu PVA and Liu D, 2009. Flavonoids and cardiovascular health. In book: Complementary and Alternative Therapies and the Aging Population. Acad Press, pp 371-392, doi: 10.1016/B978-0-12-374228-5.00018-4

Borah R and Biswas SP, 2018. Tulsi (*Ocimum sanctum*), excellent source of phytochemicals. IJEAB, 3(5): 265258, doi: 10.22161/ijeab/3.5.21

Boskabady MH, Alitaneh S and Alavinezhad A, 2014. Carumcopticum L.: A herbal medicine with various pharmacological effects. Biomed Res Int, 2014: 569087, doi: 10.1155/2014/569087

Dabeek WM and Marra MV, 2019. Dietary quercetin and kaempferol: bioavailability and potential cardiovascular-related bioactivity in humans. Nutrients, 11(10): 2288, doi: 10.3390%2Fnu11102288

Ghosh M, 2007. Fundamentals of experimental pharmacology. Indian J Pharmacol, 39(4): 216-216

Konan AML, Golly KJ, Kra AKM, Adima AA and Lohoues EEC, 2022. Phytochemical screening and toxicity assessment of *Imperata cylindrica* (L.) P. Beauv. (Poaceae) raw extracts with brine shrimp (*Artemia salina*) lethality assay. J Biosci Med, 10(8): 153-171, doi: 10.4236/jbm.2022.108014

Martinez M, Del Ramo J, Torreblanca A and Diaz-Mayans J, 1999. Effect of cadmium exposure on zinc levels in the brine shrimp *Artemia parthenogenetica*. Aquaculture, 172(3-4): 315-325, doi: 10.1016/S0044-8486(98)004311

Mc Laughlin JL, Chang CJ and Smith DL, 1991. Bench–top bioassay for the discovery of bioactive natural products. An update. In: Rhaman, A.U., Ed., Studies in Natural Products Chemistry, Elsevier, Amsterdam, pp 383-409

Meyer BN, Ferrigni NR, Putnam JE, Jacobsen LB, Nichols DJ *et al*., 1982. Brine shrimp: A convenient general bioassay for active plant constituents. Planta Med, 45(5), 31-34, doi: 10.1055/s-2007-971236

Montenegro-Landívar MF, Tapia-Quirós P, Vecino X, Reig M, Granados M *et al*., 2022. Recovery of natural polyphenols from spinach and orange by-products by pressure-driven membrane processes. Membranes, 12(7): 669, doi: 10.3390/membranes12070669

Parvez GM and Mosaddik A, 2016. Evaluation of brine shrimp cytotoxicity of mango peel and flesh after formalin treatment. IJIPSR, 4(8): 900-908

Prakash P and Gupta N, 2005. Therapeutic uses of *Ocimum sanctum* Linn (Tulsi) with a note on eugenol and its pharmacological actions: A short review. Indian J Physiol Pharmacol, 49(2): 125

Wu C, 2014. An important player in brine shrimp lethality bioassay: The solvent. J Adv Pharm Technol Res, 5(1): 57

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