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Potential cytotoxic and mutagenic effect of *Pinus wallichiana*, *Daphne oleiodes* and *Bidens chinensis*Nazish Khan^a, Ibrar Khan^{a,*}, Sadiq Azam^a, Farhan Ahmad^b, Hamid Ali Khan^c, Asma Shah^d, Midrar Ullah^e^a Centre of Biotechnology and Microbiology, University of Peshawar, Pakistan^b Department of Biotechnology, Bacha Khan University, Charsadda, Pakistan^c Institute of Biological Sciences Sarhad University of Science and Information Technology, Peshawar, Pakistan^d Department of Biotechnology, Women University Mardan, KPK, Pakistan^e Department of Biotechnology Shaheed Benazir Bhutto University Sheringil, Dir Upper Khyber Pakhtunkhwa, Pakistan

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ABSTRACT

The *Pinus wallichiana*, *Daphne oleiodes* and *Bidens chinensis* have a long history of being used traditionally for treatment of various types of disorders. Most of the uses have been without any scientific evidence and toxicological assessment. We evaluated the mutagenic and cytotoxic capabilities of various parts of *P. wallichiana*, *D. oleiodes* and *B. chinensis*. Ames *Salmonella* mutagenicity assay determined the mutagenicity activity against TA 98 and TA 100 bacterial strains of *Salmonella typhimurium* without metabolic activator S9 system. The number of mutant colonies in negative control was considered as limit to determine the mutagenicity effects of every extract. Brine shrimps lethality bioassay was used to determine the cytotoxic capabilities of the selected plants. The *P. wallichiana*, *D. oleiodes* and *B. chinensis* did not showed any mutagenic activity both for frameshift mutation (TA98) and base-pair substitution (TA100) without S9 mixture. The crude methanolic extract of *P. wallichiana* stem showed moderate cytotoxicity (53.33%) at 1000 µg/ml with LD₅₀ value 599.634. The *D. oleiodes* fruit showed a toxicity of 60% at 1000 µg/ml with LD₅₀ value 367.730. The *B. chinensis* (whole plant) showed lethality of 63.3% at 1000 µg/ml, with LD₅₀ 204.833. The absence of any mutagenic activity of crude extract of the tested plants in both bacteria strains, TA 98 and TA 100 without the S9 mix confirms the safety of these plants to the consumers.

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1. Introduction

The novel physical and chemical agents obtained from medicinal plants have a good impact on the health of human beings, however, these agents may also have a mutagenic and cytotoxic effect and can cause serious health problems (Aremu et al., 2011). Plants components mediate their effects by interacting with biological targets to provide a therapeutic effect, and is one of the major sources for providing pure chemical agents to modern (allopathic) medicine (Aggarwal et al., 2006; Salazar-Gómez et al., 2020).

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The applications of plants as prebiotic and complementary drug in the treatment of the diseases is increasing day by day (Akkol et al., 2020). The World Health Organization (WHO) reported that in the developing countries, still, 80% population is dependent on folk medicine for their health well-being (Grujicic et al., 2020). The naturally occurring synergistic effect of many plant compounds make them more effective to treat a disease (Khan et al., 2018; van Wyk and Prinsloo, 2020). Irrespective of the widespread therapeutic capability and overall acceptability of herbal preparations is restricted due to the lack of dose scheme and sufficient toxicity data (Raina et al., 2015). Therefore, therapeutic plants must be screened regarding its safety, quality and potency (Saravanan et al., 2020). Ames mutagenicity assay (Ames test) for the genotoxicity determination is the most accepted and widely used in vitro test (Abou El-Nour et al., 2010). A study investigated *Agrimonia* and *Filipendula* extracts using Ames *Salmonella*/microsome test. *Agrimonia* and *Filipendula* extracts results revealed absence of reverse mutation in bacterial test strains (Pukalskienė et al., 2018). In another study twenty-two plant species were investigated for their genotoxicity

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using the Ames (*Salmonella typhimurium* strains TA98 and TA100). Dichloromethane and methanolic extracts of various plants among the selected samples had average to significant antimutagenic potential in Ames assay (Makhuvele et al., 2018). In one of the study 478 plant species were screened for possible mutagenic potential. It was identified that 388 species out of these 478 were non-mutagenic (da Silva Dantas et al., 2020). In vitro mutagenicity and in vivo genotoxicity of *V. officinalis* leaves aqueous extract were evaluated using a modified Ames test. Ames test (with and without metabolic activation) revealed that 1.25, 5, 2.5 and 0.625 mg/ml of *V. officinalis* extract produced a substantial mutagenic activity against TA98 and TA100 bacterial strains (Fateh et al., 2019). In one of the study brine shrimp lethality activity screened the aqueous extracts of 120 medicinal plants for their cytotoxic potential. Among the 120 plants tested, *Pistacia lentiscus* exhibited effective cytotoxicity with LC50 2.5 µg. *Boswellia serrata* (Burseraceae), *Ginkgo biloba* (Ginkgoaceae), *Aristolochia indica* (Aristolochiaceae), *Semecarpus anacardium* (Anacardiaceae) and *cambogia* (Clusiaceae), LC50 values also revealed the significant cytotoxicity against brine shrimps (Krishnaraju et al., 2005). Another study revealed that *S. grandiflora* leaves possessed significant cytotoxic activity when tested in vitro. The ethanolic extract of *S. grandiflora* possessed marked cytotoxic effect (Sathasivam and Lakshmi, 2017).

Pinus wallichiana A. B. Jacks (family: Pinaceae) commonly known as Himalayan blue pine is a cone-bearing evergreen tree mostly found in the North Pakistan. It is an elevated Pine, mostly ranging from 1800 to 4000 m (Khan et al., 2020). The plant resin is used as an intoxicant and for the cure of stomachic and gonorrhoea. It is externally used as blisters for suppuration and buboes plaster. The wooden part of *P. wallichiana* is useful for cough, ulceration, burning sensation of body and fainting (Nazish et al., 2018).

Daphne oleiodes Schreb. (family: Thymelaeaceae) also known by its common name olive-leaved daphne, is an evergreen shrub. It is usually about 30–50 cm in height. Several species of *D. oleiodes* have been found at subtropical and temperate Asia, Australia, North Africa, Europe and pacific regions (Riaz et al., 2016). Traditionally it is used to treat several diseases like gonorrhoea and abscesses, the root as a laxative, the bark and leaves in skin infections (Baquar, 1989). The fresh stem of the plant is used to reduce inflammation while, the aerial parts and roots are used in the treatment of diseases like lumbago and rheumatoid arthritis (Yeşilada et al., 2001). *Daphne oleiodes* have spasmolytic and spasmogenic activities and is also used for gastrointestinal motility disorders (Khan et al., 2011).

Bidens chinensis L. (family: Asteraceae) commonly known as stickseeds or beggarticks is an annual herb used in many local medicines across the world. In Pakistan, the weed is termed as Dimpal, and is taken as an invasive weed of many crops including *Triticum aestivum* and *Avena sativa* (Ullah et al., 2014). This plant has been specifically used for many inflammatory diseases of eyes and ears in several regions of the world. The major chemical constituents of the plant extracts are Phenylpropanoid glucosides, polyacetylenes, diterpenes, flavonoids and flavone glycosides that are reported to be bioactive. Majority of these compounds showed antioxidant (Chiang et al., 2004), antibacterial and antimicrobial activities (Rabe and Van Staden, 1997). The extracts of the plants are widely used in traditional Taiwan as herbal tea believed to be preventive in inflammation and cancer (Yang et al., 2006).

Irrespective of the traditional medicinal importance of these plants there might be some possible risks associated with consumption of these plants. Keeping in view the possible risks, we measured the cytotoxic and mutagenic potential of various parts of these medicinal plant using Ames test (*Salmonella* mutagenicity assay) and brine shrimps lethality bioassay. In this study we assessed these plants to evaluate the threats associated with different form of amalgams existing in these plants. These kind of studies allows to con-

clude the threats associated to the application of these plants as conventional medicine and thus, this work would give standard baseline information on these plants that may possibly be used as a basis for the therapeutically important new tools development.

2. Material and methods

2.1. Plants collection, extraction and fractionation

The selected parts (leaves, stem and resin) of *P. wallichiana* (stem, fruit and roots) of *D. oleiodes* fruit and *B. chinensis* were collected from Malamjabba, Khyber Pakhtunkhwa, Pakistan. The taxonomic classification was provided by Dr. Lal Badshah, Botany Department, University of Peshawar (Voucher # COBAM/Bot-13) and were also confirmed by The Plant List (2013). The dried samples were grounded to powder followed by soaking (twice) in methanol at room temperature for 15 days with proper shaking. The soaked materials were filtered each time and concentrated using rotary evaporator at 40 °C for obtaining Crude Methanolic Extract (Cr. Met. Ext). The Cr. Met. Ext of each part was preserved for various biological activities (Nazish et al., 2018).

2.2. Ames mutagenicity test

Mutagenicity test were performed using commercial test kit Muta-Chromate without metabolic activation using an authorized bacterial reverse-mutation assay (Ames assay) in liquid culture (fluctuation test) as shown in Fig. 1. Two mutant strains i.e. TA100 (*Salmonella typhimurium*) and TA98 (*S. typhimurium*) were used; TA98 was used to evaluate frame shifts while TA100 determines the base pair exchanges. Sterile distilled water, test samples (plant extracts), reagent mixture and standard mutagen were mixed in bottles by the amount shown in Table 1. For each plant extract, three different concentrations (50, 500 and 5000 µg/ml) were used and mutagenicity test were performed in triplicates. The sodium azide was administered as positive control for TA100 and 2-nitro fluorine for TA98 without metabolic activation. After inoculation of TA100 and TA98, incubation is done in nutrient broth for 18–24 h at 37 °C. Rigorous mixing of culture broth (5 µl) was followed by shifting of every bottle content to multi-channel reagent boat and 200 µl of mixture were transferred by multichannel pipette in 96-well micro-titration plate. Plates were incubated at 37 °C for 4 days in airtight plastic bag to avoid evaporation. After incubation revertant colonies were counted using binocular microscope in comparison to control. Blank plate was examined first and when blank plates showed purple coloration then rest of the plates were observed as this specified that the test was not contaminated. All the test samples were marked toxic to the bacteria, if total wells in test plate specified purple color. If positive wells number exceeds twice the number of positive wells in background plate (spontaneous mutation), the test samples were considered mutagenic (Razak and Aidoo, 2011).

Salan statistical software was used to analyze number of revertants. The Mutagenicity Index is the difference of average number of mutant colonies/plate to the average number of mutant colonies/plate from the negative control. The test sample was marked positive if the MI ≥ 2 for at least one of the tested doses and if it gave a consistent dose–response curve. Extracts concentrations showing significant difference in revertants frequency in contrast to negative control with MI higher than 1.5 and lower than 2 were signified as a weak mutagen (Santos et al., 2010).

2.3. Cytotoxicity test (Brine shrimp lethality assay)

Brine shrimp lethality bioassay (Krishnaraju et al., 2006) was used to determine the cytotoxic capability of the *P. wallichiana* stem,

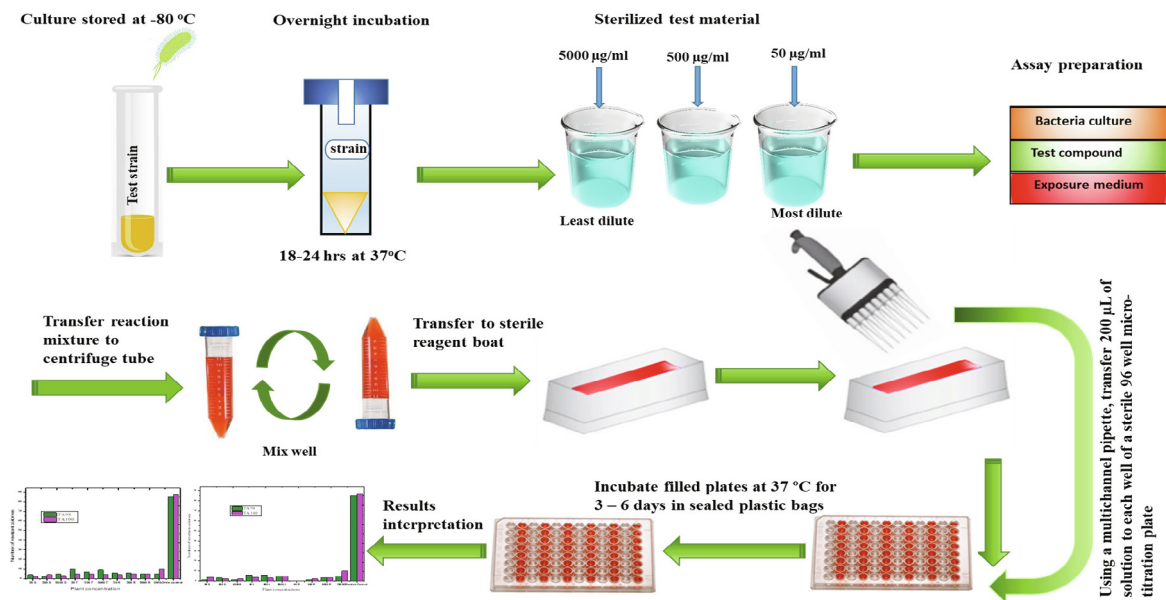


Fig. 1. Ames bacterial reverse-mutation in liquid culture (fluctuation test).

Table 1
Set up of the Ames assay.

Treatment	Volume added (µl)				
	Mutagen Standard	Sample	Reagent Mixture	Deionized water	Salmonella test strain
Blank	–	–	2.5	17.5	–
Background	–	–	2.5	17.5	0.05
Standard mutagen	0.1	–	2.5	17.4	0.05
Test Sample	–	0.005	2.5	17.5	0.05

D. olooides fruit and *B. chinensis* (whole plant) extracts using *Artemia salina* (test organism). The Fig. 2 represents the entire assay used in this experiment. Artificial sea water was composed of commercial salt mixed with double distilled under continuous aeration for 48 h. Brine shrimp eggs were hatched in conical shaped vessel (1L) filled with sterile artificial seawater. After hatching the active nauplii were collected for the test. Then stock solution was prepared by dissolving 10 mg of plant extract in 1 mL of distilled water. Concentrations prepared by serial dilution from the stock solution (10, 100 and 1000 µg/ml) were added to the pre-labelled test tubes. Then from each concentration 1 mL solution was added into the test tubes having 1 mL of seawater and 30 nauplii. The mortality rate was evaluated after 24 h by measuring the number of dead nauplii. The Etoposide, a standard drug was used as positive control.

2.4. Statistical analysis

The percent cytotoxicity was measured by comparing the mortality mean in the control and test tubes. LC₅₀ values were calculated from the best-fit line plotted concentration verses percentage. Extracts resulting from natural products are investigated to have toxic properties if they are having LC₅₀ ≤ 1.0 mg/mL.

3. Results

3.1. Mutagenicity test (Ames assay)

The results of mutagenicity test were based on the total number of mutant colonies of both bacterial strains (TA98 and TA100) in contrast to the -ive control (background plate). The blank plate was read first, rest of the plates were then examined when entire

wells in the blank plate were stained purple, specifying that the assay was not contaminated. The mutagenicity analysis of *P. wallichiana* (stem, leaves and resin), *D. oleoides* (stem, root and fruit), and *B. chinensis* (whole plant) is given in Figs. 3-5 against the selected strains. The positive and negative controls showed results in the normal range found in the laboratory. In TA 98 strain the maximum number of mean mutant colonies per plate were detected at the dosage of 50 g/ml *D. oleoides* fruit extract in the absence of metabolic activator however, this mutation frequency did not change significantly for both strains in comparison to spontaneous mutation frequency (p greater than 0.05) as indicated by one-way ANOVA test. In comparison to the negative control none of the test sample showed double or more than double the number of mutant colonies showing that none of the samples possess mutagenic potential and were non mutagenic. The Mutagenicity Index (MI) for each dose, at highest concentration of plant extracts, was calculated as mentioned in table 2.

3.2. Brine shrimp lethality bioassay

Brine shrimp (*Artemia salina*) lethality bioassay was used to determine the cytotoxic potential of the test samples. The results of the assay are stated in Fig. 6. The results revealed that the Cr. Met. Ext of *P. wallichiana* stem showed moderate cytotoxicity (53.33%) at 1000 µg/ml. At 100 and 10 µg/ml, lethality was 40 and 33.33%, respectively. The LD₅₀ value was 599.634. The *D. oleoides* fruit showed a toxicity of 60% at 1000 µg/ml and 36.66 and 20%, at 100 and 10 µg/ml respectively. The LD₅₀ value recorded was 367.730. The *B. chinensis* (whole plant) showed lethality of 63.3% at 1000 µg/ml. It showed cytotoxicity of 43.33 and 26.66% at 100 and 10 µg/ml with LD₅₀ 204.

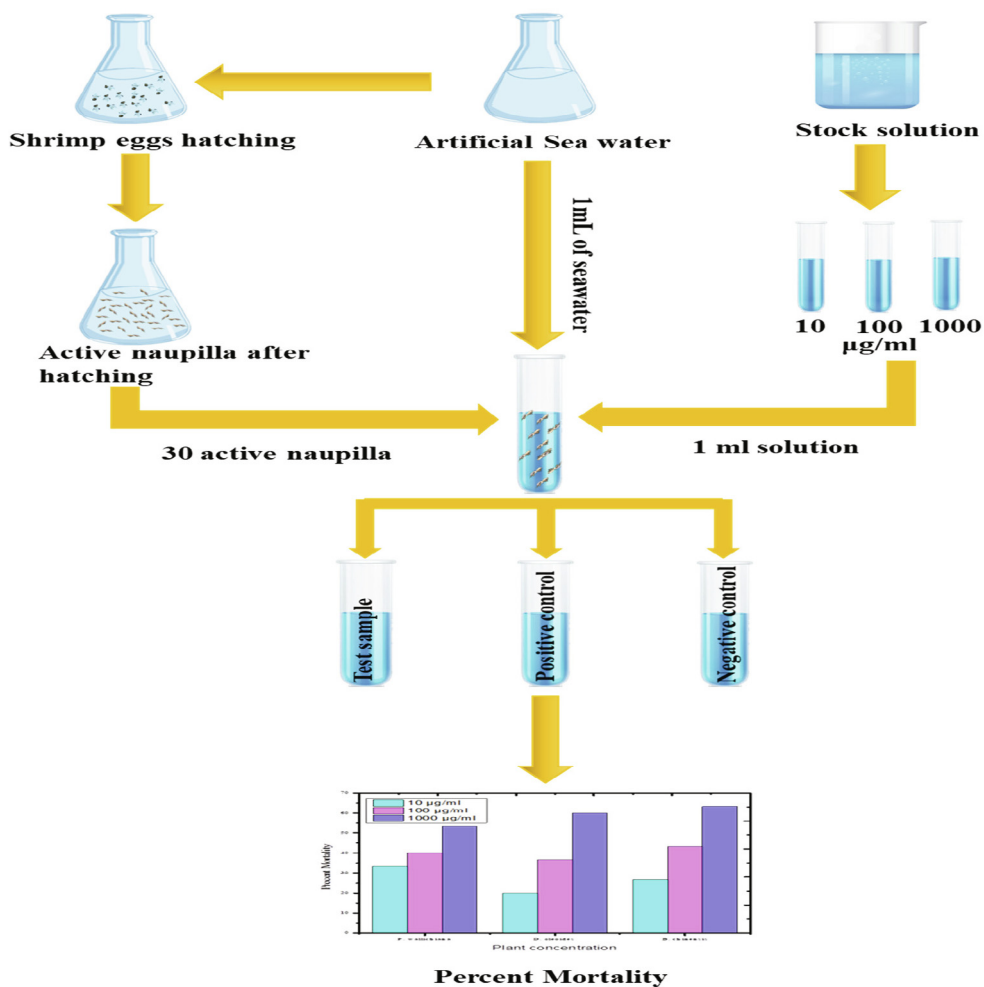


Fig. 2. Brine Shrimp Lethality Assay of plant extracts.

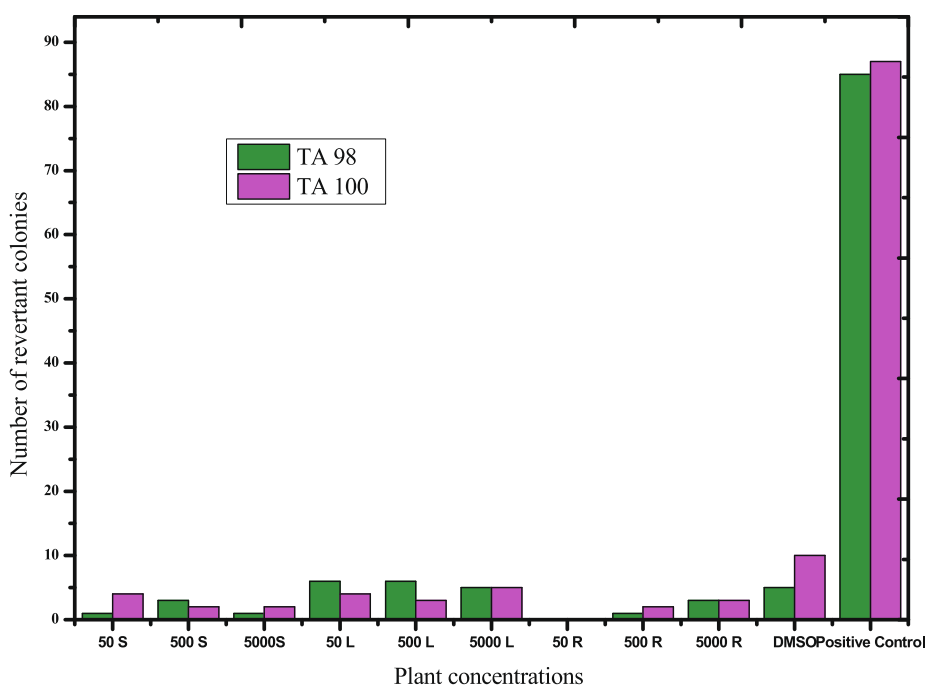


Fig. 3. Mutagenicity analysis of *P. wallichiana* stem, leaves and resin.

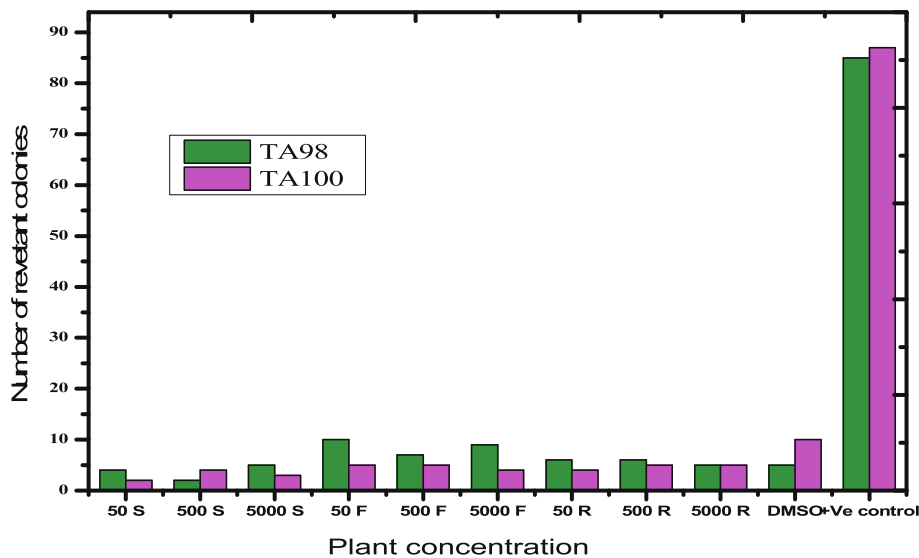


Fig. 4. Mutagenicity analysis of *D. oleoides* stem, root and fruit.

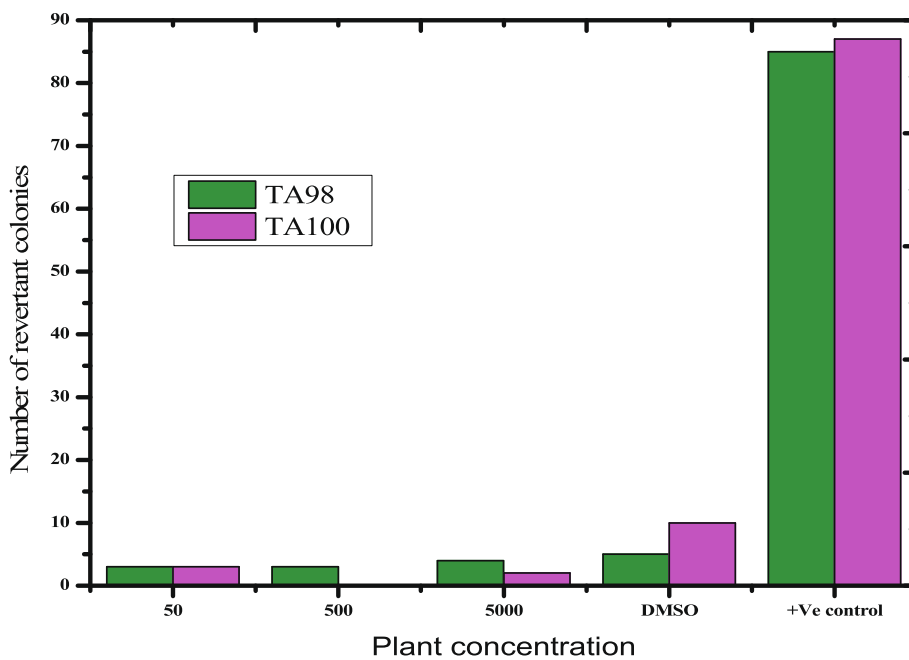


Fig. 5. Mutagenicity analysis of *B. chinensis* whole plant.

Table 2

Mutagenicity index at highest concentration (5000 µg/ml) of *P. wallichiana* (stem, leaves and resin), *D. oleoides* (stem, root and fruit), *B. chinensis* (whole plant).

Plant specie	TA98		TA100	
	Mutagenicity index (MI)	Results	Mutagenicity index (MI)	Results
<i>P. wallichiana</i>	Stem	0.2	Non-mutagenic	Non-mutagenic
	Leaves	1	Non-mutagenic	Non-mutagenic
	Resin	0.6	Non-mutagenic	Non-mutagenic
<i>D. oleoides</i>	Stem	1	Non-mutagenic	Non-mutagenic
	Fruits	1.8	Non-mutagenic	Non-mutagenic
	Roots	1	Non-mutagenic	Non-mutagenic
<i>B. chinensis</i>	Whole plant	0.8	Non-mutagenic	Non-mutagenic

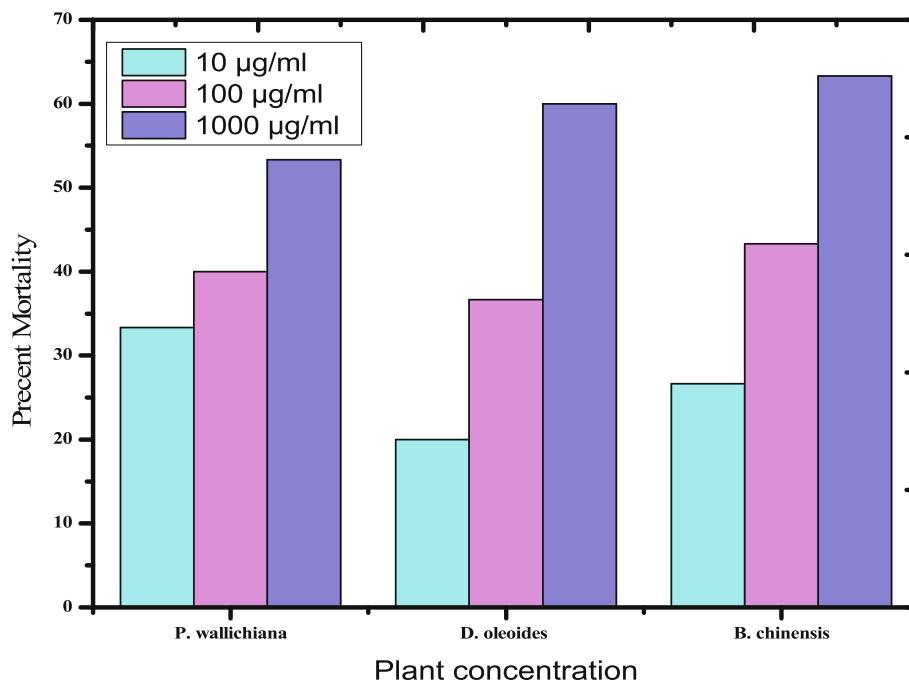


Fig. 6. Percent brine shrimp mortality of *P. wallichiana* stem, *D. oleoides* fruit, *B. chinensis* (whole plant).

4. Discussions

The use of medicinal plants have been considered as a part health care of human culture but some the researches presented the carcinogenicity and mutagenicity of various therapeutically important plants (Akintonwa et al., 2009). The keen interest of many researchers on natural constituents has been increasing every passing day (Eren and Özata, 2014), thereby the assessment of mutagenicity and cytotoxicity of these constituents is very important. The reported literature survey suggests that the mutagens having carcinogenic nature ranges from 50 to 90% so, the Ames test is suggested for initial screening of medicinal plants. The positive results in any case (single bacterial strain) is sufficient to identify the presence of mutagen. The results of present study indicated the absence of mutagenic substances in the crude methanolic extracts of these three plant species used in Ames assay directly (without metabolic activation) thus, the revertant colonies of both bacterial strains TA98 and TA100 are very specific to certain chemicals constituents that acts using this procedure. The lack of a mutagenic potential of these plants against T98 and TA100 strains in the Ames assay is the first positive step, to the best of our knowledge, about the non-mutagenic nature of these three selected plants, which is very important since these plants has shown several therapeutic properties.

According to the reported study the aglycone quercetin and amentoflavone with and without metabolic activation possessed strong mutagenic activity to the TA98 test strain (Cardoso et al., 2006). In another study, the Ames test determined the mutagenicity of the hydroalcoholic extract of *Ocotea duckei* Vattimo and of yangambin. Only *Ocotea duckei* Vattimo hydroalcoholic extract gives positive results. While the Yangambin was not mutagenic (Marques et al., 2003). The non-mutagenic potential of various plant species reported in literature along with our findings promotes the safer use of these plants for medicinal purposes. The current study revealed the first evidence about the non-mutagenic nature of the selected plant species used in this study.

Brine shrimp lethality bioassay is an easy, useful and appropriate screening process for greater number of dilutions and samples

within shorter time to assess the plant extracts lethality to brine shrimp (Krishnaraju et al., 2006). The results of current study revealed that the methanolic extracts of the tested medicinal plants were effective towards brine shrimps however Cr. Met. Ext of *B. chinensis* was the most active at 1000 µg/ml. All the test samples showed maximum lethality at higher concentration of 1000 µg/ml. All the observed results suggested the concentration dependent cytotoxic activity of plant extracts. The experimental cytotoxicity of the tested plant decoctions to brine shrimps specified the availability of effective cytotoxic and certainly antitumor constituents. Meyer et al. (Olowa and Nuñez, 2013), declared toxicity of plant crude extract if it has an LC50 value of less than 1000 µg/mL while if it is greater than 1000 µg/mL then they are considered non-toxic (inactive). The cytotoxic ability of plant extracts can be linked to the presence of secondary metabolites detected in these plants through preliminary phytochemical screening tests. As in a study reported by Vital and Rivera suggested, the expected antimicrobial activity of *C. odorata* was owing to its binding capability to bacterial cell wall, thus preventing its formation. This inhibitory action might be attributed to the presence of the tannins and flavonoids in the plant (Vital and Rivera, 2009). The researchers attributed the cytotoxic capability of plants to the presence of secondary metabolites (Chakraborty et al., 2011). Natural products needs assessment to be used as homoeopathic drug, regarding their pharmaceutical effects, lethality, dosage, and extent of treatment to determine its safer consumption.

5. Conclusion

The absence of any mutagenic activity in both bacteria strains, TA 98 and TA 100 without the S9 mix of crude extract of the three plants used proposes that the random use of domestic remedies of these plants can be safe to the consumers. Natural products needs assessment to be used as homoeopathic drug, regarding their pharmaceutical effects, lethality, dosage, and extent of treatment to determine its safer consumption.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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