To study the mutagenic potential of Artavol® using the Ame's Test by Microbial assay – A research article

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ABSTRACT.

Natural products continue to play an important role in the discovery and development of new pharmaceuticals. Several chemical compounds have been extracted and identified from its species The increasing use of herbal medicinal items calls for safety testing to protect the public from unintended hazardous effects. However, most are not tested, putting the public at risk. The modified Ames test is a useful resource for determining the mutagenic potential of medicinal products and was used this study to determine the safety of an herbal tea.In the present experiment we are discussing The Ames test has become one of the most commonly used tests to assess the mutagenic potential of medicinal plants since they have several biological activities and thus have been used in traditional medicine and in the pharmaceutical industry as a source of raw materials. Accordingly, this review aims to report previous use of the Ames test to evaluate the mutagenic potential of medicinal plants. However, the reliability of many reviewed studies regarding the botanical extracts may be questioned due to technical issues, such as testing being performed only in the presence or absence of S9, use of maximum doses below 5 mg/plate and lack of information on the cytotoxicity of tested doses. These methodological aspects additionally demonstrated that a discussion about the doses used in research on mixtures,

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INTRODUCTION

Ame's test commonly referred to as the bacterial reverse assay test is a test that was developed by Bruce Ames in1973 to detect the ability of a chemical(compounds)to induce mutation in Salmonella typhimurium1.Anychemicalsubstance(compounds)thatiscapableofcausingtheorganismtomutate is considered mutagenic and possibly carcinogenic. Evaluation of herbal medicine products which contains polychemical substances (compounds) for their mutagenic potential has not been a common practice but of late, several products have been evaluated for their mutagenic potentials 2–10.

The Ames test was standardized in the 1970s [1] to assess the mutagenic potential of chemicals. However, in recent years, the number of studies using this assay to evaluate the mutagenic potential of plant extracts has increased. Sponchiado et al. [2], in a study on the main tests that assess the mutagenic potential of medicinal plants, found that the Ames test is one of the most commonly used, either to support the safe use of plants or to be included in the battery of preclinical trials on herbal drug production. were tested for mutagenicity by the Ames test using Salmonella typhimurium TA 97a, TA 98, TA 100, and TA 104 strains, with and without metabolic activation. The genotoxicity assessment of these medicinal plants was performed in aqueous extracts 1:5. Seventy percent of these herbs presented mutagenic effects with at least one of the Ames strains used in this study. Bauhinia variegata L., E. macrophyllum K., and M. chamomilla L. showed no mutagenic activity. The mutagenic effects were detected mainly with the strains TA 98 related to frameshift mutations. Although many people believe that herbal ,are view study conductedin 2020 on 488 medical plants indicated that 98 of those on which Ame's tests were done demonstrated mutagenic potentials, 83 antimutagenic potentials and 388 were non-mutagenic. Studies conducted on four herbalmedicinalplants in Saudi Arabia indicated that herbalmedicinalplants may be safe at lower doses but become mutagenic at higher doses 6. This and many more studies on herbal medicinal products that have indicated their mutagenic potential is an indication that herbal medicine products need to be evaluated for their mutagenic potential before being allowed for sale to the general public. In this current study, we conducted a mutagenicity experimental study on Artavol® to determine its mutagenic potential since the product has not been studied for its mutagenic effects yet it is an important malaria preventive herbal tea being used population in india.

Materialsandmethods.

This was an experimental study conducted over a Microbiology Laboratory ,Department of Microbiology, Osmania University ,Hyderabad india.

Materials used: The Ames test uses several strains of the bacterium Salmonella typhimurium that carry mutations in genes involved in histidine synthesis. These strains are auxotrophic mutants, i.e. they require histidine for growth, but cannot produce it. The method tests the capability of the tested substance in creating mutations that result in a return to a "prototrophic" state, so that the cells can grow on a histidine-free medium.

The tester strains are specially constructed to detect either frameshift (e.g. strains TA-1537 and TA-1538) or point (e.g. strain TA-1531) mutations in the genes required to synthesize histidine, so that mutagens acting via different mechanisms may be identified. Some compounds are quite specific, causing reversions in just one or two strains.[4] The tester strains also carry mutations in the genes responsible for lipopolysaccharide synthesis, making the cell wall of the bacteria more permeable,[5] and in the excision repair system to make the test more sensitive.[6]

Larger organisms like mammals have metabolic processes that could potentially turn a chemical considered not mutagenic into one that is or one that is considered mutagenic into one that is not.[7] Therefore, to more effectively test a chemical compound's mutagenicity in relation to larger organisms, rat liver enzymes can be added in an attempt to replicate the metabolic processes' effect on the compound being tested in the Ames Test. Rat liver extract is optionally added to simulate the effect of metabolism, as some compounds, like benzo[a]pyrene, are not mutagenic themselves but their metabolic products are.[3]

The bacteria are spread on an agar plate with a small amount of histidine. This small amount of histidine in the growth medium allows the bacteria to grow for an initial time and have the opportunity to mutate. When the histidine is depleted only bacteria that have mutated to gain the ability to produce its own histidine will survive. The plate is incubated for 48 hours. The mutagenicity of a substance is proportional to the number of colonies observed.

PreparationofArtavol®:

Preparation of crude ARTAVOL extracts ARTAVOL® extract was prepared using infusion method in which 60 mL of distilled water was boiled at 80°C, poured into 173 g powdered ARTAVOL® , and allowed to stand for 15 min. Thereafter, the extract was filtered, concentrated in vacuo at 55°C and freezedried. The aliquot of the stock solution of 5 mg/mL concentration was prepared and stored in a fridge below 4°C. The test doses of 15, 30, and 60 mg/kg was separately determined from the stock solution to have a final volume of 60, 120 and 240 µL and administered orally

Incubation of cells

Microbial cells were washed twice by adding cell staining buffer and centrifuged at 1800 rpm for 5 min. The cells were acquired on a 19 color CytofleX LX flow Cytometer (Beckman coulter, New Jersey, USA). At least 100,000 events were recorded for analysis. Gating was standardized and set using fluorescence minus one control (FMOs). Intracellular staining of cytokines Following surface staining, the cells (splenocytes) were fixed by adding 0.5 mL of fixation buffer to each sample tube, incubated in the dark for 20 min at room temperature and then centrifuged at 1800rpm for 5 min and the supernatant discarded. The fixed cells were permeabilized by re-suspending in intracellular staining perm wash buffer (diluted to 1X in deionized water) and centrifuged rpm for 5 min. A cocktail of intracellular cytokine antibodies was prepared by adding 1ul of each that is, IFN-γ BV605 (Cat. No 505839), TNF-αBV650 (Cat. No 506333), IL-2 BV785 (Cat. No. 503843), IL-4 PerCP Cy5.5 (Cat. No 504123), IL-10PE (Cat No. 505008), and IL-17A Alexa F700 (Cat. No 506914) in the tube. The antibody cocktail was added to each of the sample tubes and incubated for 20 min in the dark at room temperature. Cells were washed 2 times with 2 mL of Intracellular Staining Perm Wash Buffer and centrifuged at 1800 rpm for 5 min. The cells were then resuspended in 200 µL Cell Staining Buffer. The samples were acquired on the Cytoflex LX flow cytometer. Compensations and fluorescent minus ones (FMOs) controls for each of the antibody fluorochromes were ran together with the optimization gains for every channel using unstained cells. Compensation calculations were done and applied to the

samples. At least one hundred thousand events were acquired and recorded per sample. Data was exported for analysis using flowJo software. Statistical analysis Flow cytometry data were analyzed using FlowJo version 10.7.1 software (Becton Dickinson, New Jersey, USA). Statistical data were analyzed using Graph Pad Prism version 8.0.3 (Prism, 2018). Lymphocytes were carefully gated on a forward scatter and side scatter plot. The percentage of positive cells for each marker was determined on the basis of the numbers of viable lymphocytes. The frequencies of T cells expressing activation and memory markers were measured as a percentage of the CD4+ and CD8+ T cell parent cell populations. Bivariate dot plots or probability contour plots were generated upon data analysis to display the frequencies of and patterns by which individual cells co-express certain levels of cell surface antigen and intracellular cytokines. Results obtained were expressed as mean ± standard error of mean. Differences in the frequencies of the T cell subsets were evaluated using a Oneway Analysis of Variance while the difference among the means was considered at 95% confidence interval using the post-hoc method of Dunnett's Multiple Comparison Test.

Results

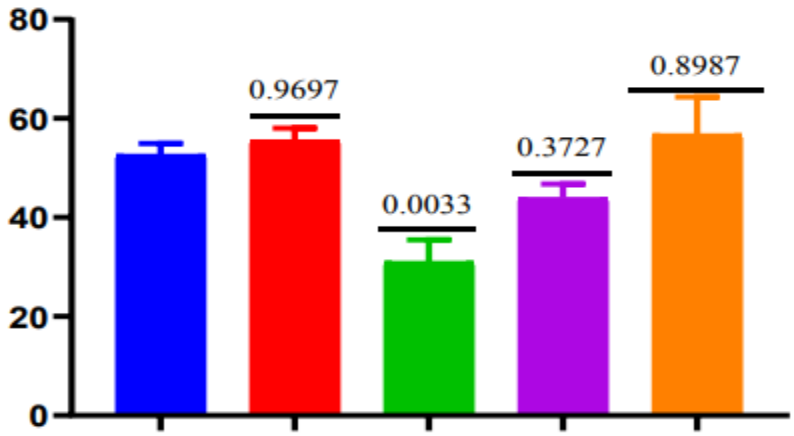


Fig 1

Mutagens identified via Ames test are also possible carcinogens, and early studies by Ames showed that 90% of known carcinogens may be identified via this test.[8] Later studies however showed identification of 50–70% of known carcinogens.[citation needed] The test was used to identify a number of compounds previously used in commercial products as potential carcinogens.[9] Examples include tris(2,3-dibromopropyl)phosphate, which was used as a flame retardant in plastic and textiles such as children's sleepwear,[10] and furylfuramide which was used as an antibacterial additive in food in Japan in the 1960s and 1970s. Furylfuramide in fact had previously passed animal tests, but more vigorous tests after its identification in the Ames test showed it to be carcinogenic.[11] Their positive tests resulted in those chemicals being withdrawn from use in consumer products.

One interesting result from the Ames test is that the dose response curve using varying concentrations of the chemical is almost always linear,[8] indicating that there is no threshold concentration for mutagenesis. It therefore suggests that, as with radiation, there may be no safe threshold for chemical mutagens or carcinogens.[12][13] However, some have proposed that organisms could tolerate low levels of mutagens due to protective mechanisms such as DNA repair, and thus a threshold may exist for certain chemical mutagens.[14] Bruce Ames himself argued against linear dose-response extrapolation from the high dose used in carcinogenesis tests in animal systems to the lower dose of chemicals normally encountered in human exposure, as the results may be false positives due to mitogenic response caused by the artificially high dose of chemicals used in such tests.[15][16] He

also cautioned against the "hysteria over tiny traces of chemicals that may or may not cause cancer", that "completely drives out the major risks you should be aware of".[17]

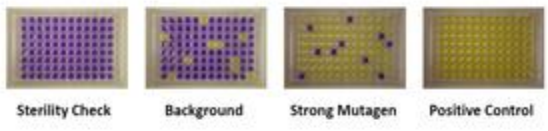
The Ames test is often used as one of the initial screens for potential drugs to weed out possible carcinogens, and it is one of the eight tests required under the Pesticide Act (USA) and one of the six tests required under the Toxic Substances Control Act (USA).[18]

Limitations

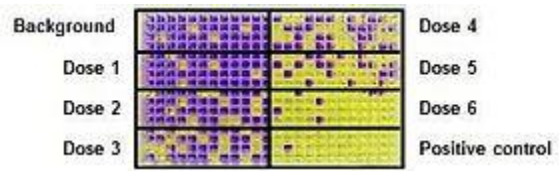
Salmonella typhimurium is a prokaryote, therefore it is not a perfect model for humans. Rat liver S9 fraction is used to mimic the mammalian metabolic conditions so that the mutagenic potential of metabolites formed by a parent molecule in the hepatic system can be assessed; however, there are differences in metabolism between humans and rats that can affect the mutagenicity of the chemicals being tested.[19] The test may therefore be improved by the use of human liver S9 fraction; its use was previously limited by its availability, but it is now available commercially and therefore may be more feasible.[20] An adapted in vitro model has been made for eukaryotic cells, for example yeast.

Mutagens identified in the Ames test need not necessarily be carcinogenic, and further tests are required for any potential carcinogen identified in the test. Drugs that contain the nitrate moiety sometimes come back positive for Ames when they are indeed safe. The nitrate compounds may generate nitric oxide, an important signal molecule that can give a false positive. Nitroglycerin is an example that gives a positive Ames yet is still used in treatment today. Nitrates in food however may be reduced by bacterial action to nitrites which are known to generate carcinogens by reacting with amines and amides. Long toxicology and outcome studies are needed with such compounds to disprove a positive Ames test.

Fluctuation method



Fluctuation method: 96-well plate



Fluctuation method: 384-well plate

The Ames test was initially developed using agar plates (the plate incorporation technique), as described above. Since that time, an alternative to performing the Ames test has been developed, which is known as the "fluctuation method". This technique is the same in concept as the agar-based method, with bacteria being added to a reaction mixture with a small amount of histidine, which allows the bacteria to grow and mutate, returning to synthesize their own histidine. By including a pH indicator, the frequency of mutation is counted in microplates as the number of wells which have changed color (caused by a drop in pH due to metabolic processes of reproducing bacteria). As with the traditional Ames test, the sample is compared to the natural background rate of reverse mutation in order to establish the genotoxicity of a substance. The fluctuation method is performed entirely in liquid culture and is scored by counting the number of wells that turn yellow from purple in 96-well or 384-well microplates.

In the 96-well plate method the frequency of mutation is counted as the number of wells out of 96 which have changed color. The plates are incubated for up to five days, with mutated (yellow) colonies being counted each day and compared to the background rate of reverse mutation using established tables of significance to determine the significant differences between the background rate of mutation and that for the tested samples.

In the more scaled-down 384-well plate microfluctuation method the frequency of mutation is counted as the number of wells out of 48 which have changed color after 2 days of incubation. A test sample is assayed across 6 dose levels with concurrent zero-dose (background) and positive controls which all fit into one 384-well plate. The assay is performed in triplicates to provide statistical robustness. It uses the recommended OECD Guideline 471 tester strains (histidine auxotrophs and tryptophan auxotrophs).

The fluctuation method is comparable to the traditional pour plate method in terms of sensitivity and accuracy, however, it does have a number of advantages: it needs less test sample, it has a simple colorimetric endpoint, counting the number of positive wells out of possible 96 or 48 wells is much less time-consuming than counting individual colonies on an agar plate. Several commercial kits are available. Most kits have consumable components in a ready-to-use state, including lyophilized bacteria, and tests can be performed using multichannel pipettes. The fluctuation method also allows for testing higher volumes of aqueous samples (up to 75% v/v), increasing the sensitivity and extending its application to low-level environmental mutagens.[21]

Discussion

This current research was conducted with the main aim of determining the mutagenic potential of Artavol® using the bacterial reverse mutation study, the modified Ames test. Findings have indicated that Artavol® is not mutagenic againstSalmonella typhimurium strains TA98 and TA100. Chemical substances tested for their mutagenic potential against strains of TA98 and TA100 are considered mutagenicaccordingtotheEBPIprotocolifgreaterorequalto25wellsoutofthe48wells(52.08%)of the cultured organisms on the positive control plate revert to positive and less or equal to zero (0) or less or equal to 15 (31.25%) wells in the negative control plates reverts to positive 17. In this study, results have indicated zero (0)wellsturning positive in the negative controlwellsas wellas inthe test wellsandupto80%reversioninTA98and70%inTA100inthepositivecontrolwellswhichisindicative thatArtavol® is not mutagenic.

Herbal medicines have been used over the years to treat malaria and have been reported to regulate the immune system in malaria (Afolayan et al., 2020; Kamau, 2022). ARTAVOL® is an herbal product used to prevent malaria in Uganda. Investigations in vivo reported the antimalarial effects of the product (Ogwang et al., 2011b). However, there was no available data on its immunomodulatory activity during P. berghei infection which has been reported in this study. In this study, a dose- dependent was observed from 15 to 60 mg/kg in the frequency of CD4+ T cells (Figure 2a). For instance, at 30 and 60 mg/kg, an insignificant frequency increase was exhibited compared to the negative control which could be from the increase in antagonizing component(s) as the concentration increased. Kurup et al. (2019) reported that drugs may induce T cell exhaustion which can down- regulate T cell function (Kurup et al., 2019). The cell surface expression of the exhaustion marker PD-1(CD279)) was assessed (Figure 2b). The herbal test doses (30 and 60 mg/kg) did not show significant increase in the frequency of CD4+ T cells expressing PD-1(CD 279). This suggests that the herbal extract in the test doses of 30 and 60 mg/kg did not induce CD4+ T cell proliferation. Konkel et al. (2010) reported that PD-1 provide inhibitory signals that dampen T cell function and restrains their clonal expansion A review of the toxicological profiles of some of the compounds identified in Artavol®(Appendix 1a- kk)has indicated that most have not demonstrated toxic effects or that there are

scanty or very little literature available showing that the compounds are mutagenic. For example natural coumarins have been reported to have shown no mutagenic effects21,22, and that little information is available about the mutagenic effects of 2,4-Di-Tert-butylphenol 23,24. Other studies have also indicated that some herbalproductsactuallyhaveantimutagenicactivities25–28.Sincestudiesontheantimutagenicactivity of artavol® was not consider in this current study, it is difficult to tell if artavol® could be having an antimutagenic activity since it demonstrated no mutagenic effects.

The Ames test 29which is a simple process of determining the mutagenic potential of compounds and thustheirpossiblecarcinogenicpotentialsandhascommonlybeenreferredtoasthetestthatchanged the world 30 should be utilized in the screening of many herbal products before being allowed in the openmarket.Assuch,itispossibletoconcludethatthechemicalcompoundsArtavol®isnotmutagenic andmaynotbecarcinogenicsinceithasdemonstratedthatitisnotmutagenicagainstbothSalmonella typhimurium TA98 and TA100 in a bacterial reverse assay test.The chemical constituents of Artavol® have not been reported to be carcinogenic and Artavol® thus, does not induce mutation either by a frameshift of base pair substitution mechanism, a mechanism that is demonstrated by test using the two strains of Salmonella if found positive.

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