Evaluation of the mutagenic potential of Artavol® using the Ame's Test.

ABSTRACT.

Background: 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 ISO test is a useful resource for determining the mutagenic potential of medicinal products and was usedthis study to determine the safety of an herbal tea like Artavol®.

Methods: This study used Ame's Modified ISO test to analyze aqueous extract of Artavol®, in a Level II biosafety cabinet. Artavol® was extracted using a decoction method, freeze dried, diluted to concentrations of 125µg, 250µg, and 500µg and used in the study. Mutagenicity was tested by culturing *Salmonella typhimurium* TA98 and TA100. Results were considered by observing colour change in the wells of the microtitre plates from purple to yellow indicating mutation. Valid results were determined by comparing negative and positive control plates after 3 days of incubation, with positive results showing a color change from purple to yellow.

Results:no colourchanges were noted in all wells containing 125µg, 250µg, and 500µg ofArtavol® up to day 3, while negative control showed color changes equivalent to 80% for TA98 and 70% for TA100.

Conclusion: Aqueous extract ofArtavol® is not mutagenic. Further safety test such as sub-chronic toxicity study ad teratogenicity studies are recommended to provide more safety data on the product.

Key Words: Artvol®, Mutagenicity, Ame's test, *Artemisia annua*, dihydroartemesinin-3-desoxy- and deoxyartemisinin, Cedrol.

Comment [PB1]: In this study

Comment [PB2]: Mention the reference

Comment [PB3]: Sentence change .: colour changes were not observed

INTRODUCTION

Ame's test commonly referred to as the bacterial reverse assay test is a test that was developed by Bruce Ames in 1973 to detect the ability of a chemical (compounds) to induce mutation in *Salmonella typhimurium*[1]. Any chemical substance (compounds) that is capable of causing the organism to mutate 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].

In Uganda, this has never been done and yet there is widespread use of herbal medicine products in the country just like in many parts of the world[11,12]. The use of these products is mostly unregulated and many are not tested for their safety either in animals or humans. Furthermore, there are several products available and allowed to be sold in the open market by the National Drug Authority with none of them meeting the standards set even by the National Drug Authority on proof of safety and efficacy[13]. This is contradicted by the presence of similar products from India and other countries where some tests on those products have been done and clinical trials conducted in whicha review report indicated that there were 234 local herbal products notified to NDA and 163 products from other countries as of June 2022[14]. Among these products wasArtavol®, a product developed from *Artemesia annua*, Lemon grass, and Avocado seed powder. The product was reported to be free of Artemisinin as it had been removed during the extraction process and was reported as safe to a limit of 5000mg/kg in a study by [15]and confirmed in another validation study [16]. In the same study, the chemical composition of the contents of Artavol® was determined by the Gas-Chromatography Mass-Spectroscopy (GC-MS) analysis and was noted to contain dihydroartemesinin-3-desoxy- and deoxyartemisinin among 40 other compounds[16]

Although many people believe that herbal medicine products are safe, a review study conducted in 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[3,5]. Studies conducted on four herbal medicinal plants in Saudi Arabia indicated that herbal medicinal plants 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 by everyone including pregnant women in Uganda.

Comment [PB4]: Use Herbal medicine rather or mention the substance name

Materials and methods.

This was an experimental study conducted over a period of 7 days in the MicrobiologyLaboratory, Department of Microbiology, Mbarara University of Scienceand Technology.

Comment [PB5]: Mention place and country : International journal than university name

Materials used: Salmonella Typhimurium TA98 and TA100, Extract of Artavol®,Falcon tubes of 15mL&50mL, micropipettes of 1000 &20-200uL(microliter) pipettes, micropipette tips, reagent boats, vortexer, liquid culture media, reversion solution (positive control), histidine, tween 80, weighing scale, Incubator, disinfectants (for cleaning the benchtop and Biosafety cabinet before

use), gloves, face mask, Level II biosafety cabinet, McFarland standard tube number 0.5, distilled water (specially supplied for Ame's test), and 96 well microtitre plates.

Source of organisms and test materials.

The test drug/herb was purchased from the manufacturer Artavol Ltd P.O Box 34 Ntinda Kampala, Plot 2 Ashok Road, Akright Estate Wakiso District.

The test organism and all reagents were purchased from the Environmental Bio-detection Product Inc (EBPI) 6800 Campobello Rd, Mississauga, Ontario, Canada L5N 2L8. All products were stored at the required temperatures untiluse.

Preparation of Artavol®:

Artavol extract was prepared by mixing the powder previously pre-extracted and used in the acute toxicity study [16]for use in this current mutagenicity test. Doses of 125µg, 250µg and 500µg were prepared and used for the test. Briefly, Artavol extract was prepared following the directives for use of the product in the packet insert, where the product is mixed with boiling water allowed to brew and taken. In this case, the product after mixing in hot water was filtered and the filtrate dried in a freeze drier to obtain a power that was dissolved and dilute to the various concentrations mentioned above and used in the Ames test.

Identification of the chemical compounds in Artavol®

This was conducted at the department of Government Analytical Laboratories in Wandegeya, Kampala following the method described in Oloro et al [16]

The Ame's test

This research work was conducted at the Microbiology Laboratory, department of Microbiology, Mbarara University of Science and Technology, following the procedure for the modified Ames ISO test, Version 1.1[17]. The test utilizes the liquid media rather than the traditional solid media method.

The liquid culture media (Nutrient broth) was prepared a day beforethe assay and incubated for 24 hours at 37°C to rule out contamination. Briefly, using an aseptic technique a bottle of nutrient broth was opened and to it was transferred 20µL of reagent V and mixed. Two separate mixtures were prepared, one for TA98 and another for TA100. The mixture was transferred to the vial of each lyophilized Bacteria (TA98 and TA100), covered and incubated at 37°C for 19 hours. Each of the mixtureswas inspected the following day for turbidity, indicating the growth of the bacteria. The aqueous extract of Artavol® was filtered using a 22µm membrane filter before the test[17]. The aqueous Artavol® samples were then prepared to the required concentrations of 125, 250 and 500 ready for the test.

Preparation of the exposure solution[17]

This was prepared by mixing 4.15mL of the exposure medium concentrate (A), 0.50mL of the 40% D-Glucose, 0.30mL of D-Biotin and 0.05mL of L-Histidine to make a total of 5mL solution mix. To this was added the test substance at different concentrations to make 125µg, 250µg and 500µg of the mixture with Artavol® and each concentration was prepared in a separate 15mL tube

Preparation of the positive control exposure medium master mix solution[17]

40% D-Glucose (Reagent B) 2.3 ml, Bromocresol (reagent C) 3.5ml, D-Biotin (reagent D) 4.65ml, and 11.65ml of 10x reversion solution (reagent H).

Dispensing of the mix to the microtitre well plates[17]

Each preparation was aseptically dispensed in a sterile reagent boat and from it they were dispensed into the microtitre plates.

To each well in the test plate was added 0.5µL of bromocresol purple and the total volume of all the mixture in ach well was made to 200µL. Each plate was sealed in a Ziploc bag and incubated for 3 days at 37°C.

On day 4, all plates were removed and inspected for colour changes. The number of wells with colour changes were counted and expressed over the total number of wells for each dose level and multiplied by 100% to obtain the percentage reversion. This was done for all dose levels of the test and control plates.

Confirmation of positive results was taken by scoring the colour changes in the plates visually, where the colours of samples in a well changed from purple to yellow indicating mutations on the following conditions

a. Average score for the negative control is ≥ 0 and ≤ 15 revertant wells per 48-well section on day 3. (an equivalent of less or equal to 31.25% change)
b. Average score for the positive control is ≥ 25 revertant wells per 48-well section on day 3. (an equivalent of a greater than 52.1% change)

Data analysis

The microtitre plates were observed daily for 3 days to determine the colour change which would result from wells where the organisms had become metabolically active as a result of mutation. The number of wells with colour changes was divided by the total number of wells at each dose level for each organism and used to calculate the percentage reversion (No. revertant wells in test/total number of wells in each test * 100%)

All wells with colourchanges were counted on day 3 and used to calculate the percentage reversion using Microsoft Excel 2016.

Limitation

There was evaporation of the media from the wells over the 3 days which to some extent could have impacted on the results. But this was carefully noted and only wells that showed relatively deep yellow colour changes were noted as real changes due to reversion.

Some non-volatile components of the extract may have not been detected by the GC-MS during analysis and thus less compounds may have been reported.

Few wells for the negative controls were used than required.

Results

Earlier GC-MS Results were partially published

A total of 37 organic compounds were detected in Artavol® as indicated below in ***(Figure 1 a-kk)***with names and chemical structures. These were initially published in a study by Oloro et al although only four compounds (deoxyartemisinin (figure 1ee), Cedrol (figure 1 hh), Dihydroartemisinin-3-desoxy- (figure 1 ii) and Coumarin (figure 1 f)) were indicated with their chemical structures [16] the list here indicates the chemical structures of all the compounds detected in Artavol®.

Mutagenicity study results

Results generally indicate that Artavol® is not mutagenic either against *Salmonella typhimurium* TA98 or TA100.**Figures 2a, 2 b and 2c**at dose levels of 125µg, 250µg and 500µgshowed no colour changes in the wells incubated with the Aratavol® at in the presence of the S9 mix. In **Figure 2d,** the positive control shows the characteristic colour changes in wells with metabolically active organisms that have undergone mutation.

Tables

PRODUCT & DOSE (µg)	**Organism used**	**No. of revertant wells**	**No. of non-revertant wells**	**Total no. wells used**	**Percentage reversion**
Artavol® 125µg	TA98	0	32	32	0
	TA100	0	32	32	0
250µg	TA98	0	32	32	0
	TA100	0	32	32	0
500µg	TA98	0	32	32	0
	TA100	0	32	32	0
Positive control	TA98	16	4	20	80
	TA100	14	6	20	70
Negative	TA98	0	04	04	0
	TA100	0	04	04	0

TABLE 1: *The table above indicates that the Flavonoid isolated from Artemisia annua has a dose-dependent mutagenic effect against Salmonella typhimurium TA98 and an effect of 12.5 % compared to the negative control.*

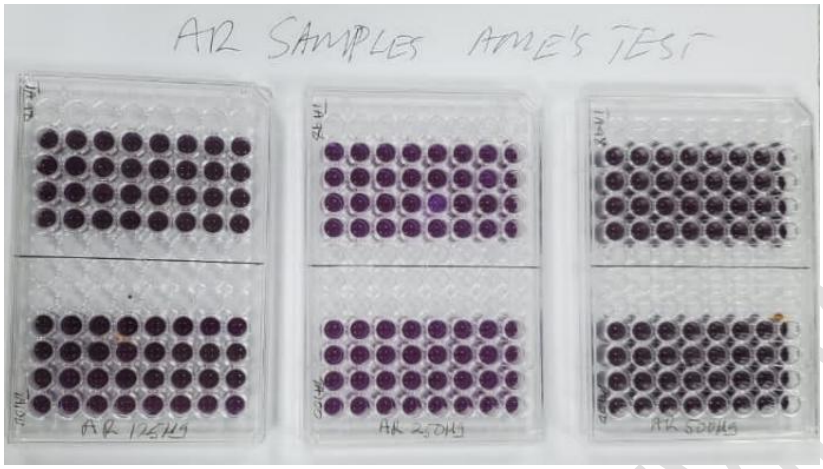

Figure 1:*Artavol® Sample 125*µg*, 250*µg *and 500*µg*on day 1 prior to incubation*

Figure 1 shows the prepared artavol® sample at 3 dose levels ready for incubatin on day 1. With same colour.

Figure 2 aArtavol 125µg on day 4

Figure 2 bArtavol 250µg on day 4

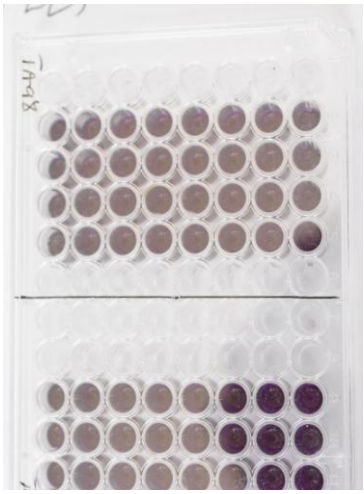

Figure 2 cArtavol 500µg on day 4

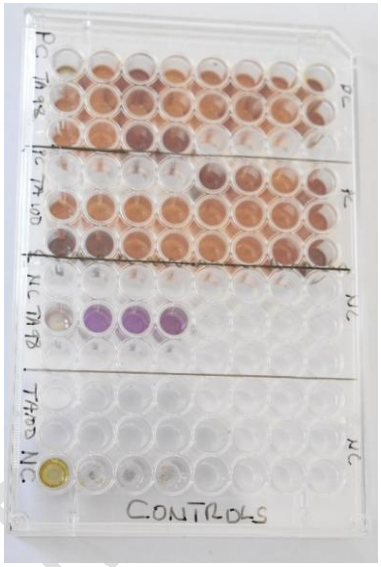

Figure 2 d control plate on day 4

Figure 2:a, b and c *Artavol® Sample* and d *Control plateon day 4 of incubation*

*Figure 2 above indicate that no colour changes took place in the wells containing varying doses of Artavol® extract (2 a. = 125*µg*, 2 b. = 250*µg *and 2 c. = 500*µg*) but positive changes occurred in the wells of the positive control plate (2 d).*

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 against*Salmonella typhimurium* strains TA98 and TA100. Chemical substances tested for their mutagenic potential against strains of TA98 and TA100 are considered mutagenic according to the EBPI protocol if greater or equal to 25 wells out of the 48 wells (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) wells turning positive in the negative control wells as well as in the test wells***(Figure 2 a-c)*** and up to 80% reversion in TA98 and 70% in TA100 in the positive control wells***(Table 1, figure 2d)*** which is indicative thatArtavol® is not mutagenic.

Previous studies conducted on the root extract of *Salacia chinensis* using Ame's test, in the presence, same method as used in our study and absence of the S9 Mix (rat liver extract after treatment with metabolic enzyme inducing drugs) indicated that the root extract was not mutagenic [7]. Experimental results conducted in the presence of the S9 mix when positive is an indication that the product under test requires metabolic activation to cause mutation and when

testing complex mixtures, the Ames miniaturized, microplate fluctuation format test (MPF) test is advantageous[18,19]. This results correspond to our findings from Artavol® which is an herbal extract (polycompound product) and did not show any mutagenic effects against both strains of salmonella TA98 and TA100. The only difference here is that, in the currentstudy, only two strains were used while in the former, five strains were used. Several studies have indicated that many polyherbal products do contain at times compounds that are mutagenic [20] and Artavol® is also a polyherbal formulation comprising products from *Artemisia annua*, avocado seeds and lemongrass. It has, however, not demonstrated any mutagenic potential, an indication that it may not contain a mutagenic compound.

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 effects[21,22], 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 herbal products actually have antimutagenic activities[25–28]. Since studies on the antimutagenic activity 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 [29]which is a simple process of determining the mutagenic potential of compounds and thus their possible carcinogenic potentials and has commonly been referred to as the test that changed the world [30] should be utilized in the screening of many herbal products before being allowed in the open market. As such, it is possible to conclude that the chemical compounds Artavol® is not mutagenic and may not be carcinogenic since it has demonstrated that it is not mutagenic against both *Salmonella 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.

Conclusion:

The current study has only proven that Artavol® is not mutagenic in the presence of the S9 mix. Studies in the absence of the S9 mix, and other toxicological tests, such as sub-chronic toxicity study and teratogenicity study should be conducted to provide a complete toxicological profiles of Aratavl®.

Comment [PB6]: Sentence change

Ethical Approvals

This study was approved by the Mbarara University of Science and Technology Research Ethics Committee with approval number MUREC 1/7 and Uganda National Council for Science and Technology with Registration number HS540ES.

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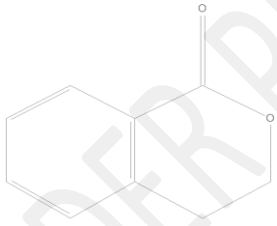
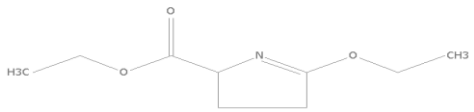
APPENDIX 1: CHEMICAL COMPOUNDS DETECETED IN ARTAVOL®[16]

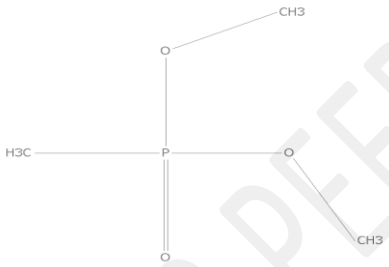
a. 1H-2-Benzopyran-1-one, 3,4-dihydro-



b. 5-Ethoxy-3,4-dihydro-2H-pyrrole-2-carboxylic acid, ethyl ester

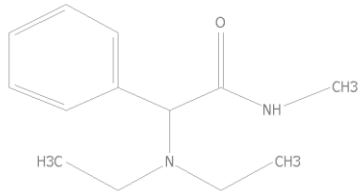


c. Dimethyl methylphosphonate



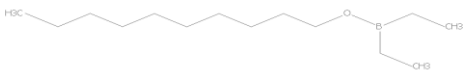
d. 7-Hydroxythujone

e. 2-Diethylamino-N-methyl-2-phenyl-acetamide

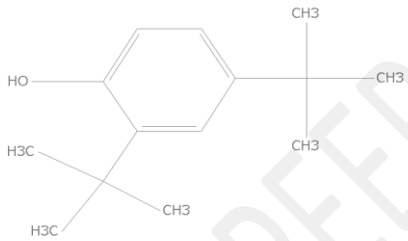


f. Coumarin

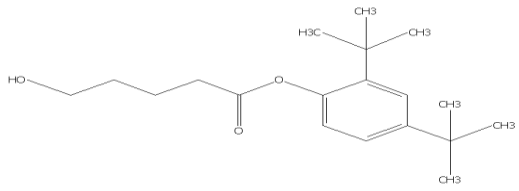
g. Borane, diethyl(decyloxy)-



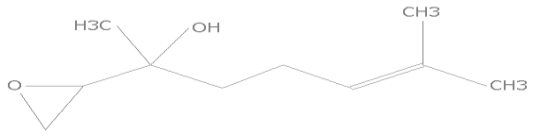
h. 2,4-Di-tert-butylphenol



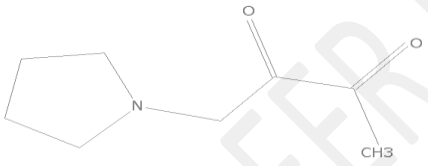
i. Pentanoic acid, 5-hydroxy-, 2,4-di-t-butylphenyl esters



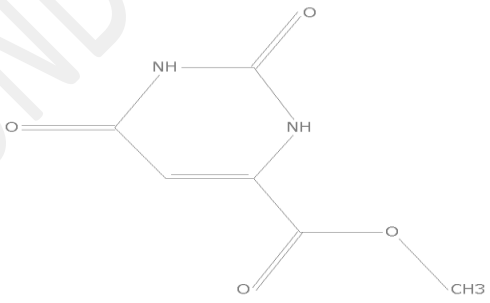
j. alpha.-Methyl-.alpha.-[4-methyl-3-pentenyl]oxiranemethanol



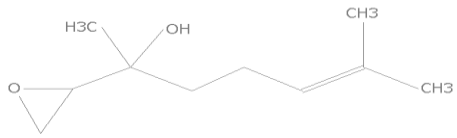
k. 2-Hydroxy-1-(1'-pyrrolidiyl)-1-buten-3-one



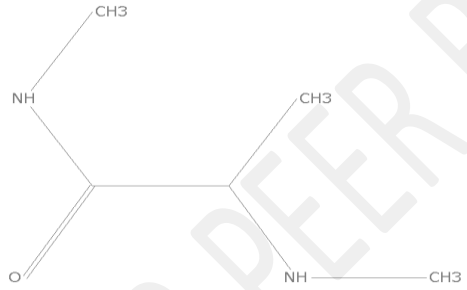
l. Methyl orotate



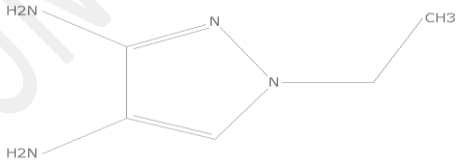
m. alpha.-Methyl-.alpha.-[4-methyl-3-pentenyl]oxiranemethanol



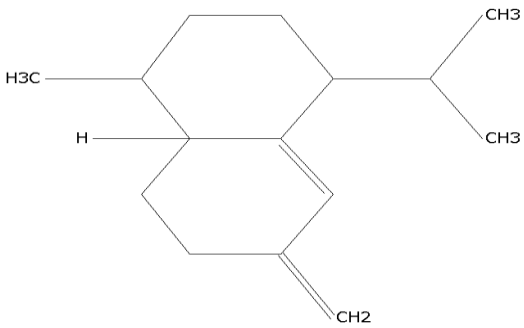
n. Propanamide, N-methyl-2-(methylamino)-



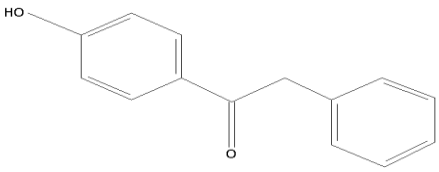
o. 1-Ethyl-1H-pyrazole-3,4-diamine



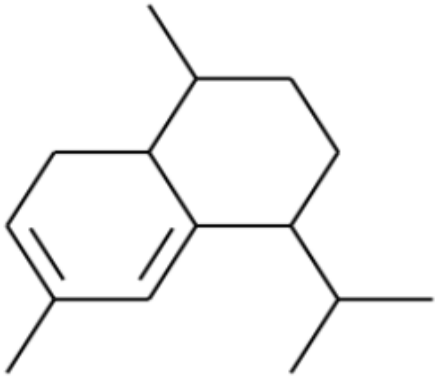
p. Bicyclosesquiphellandrene



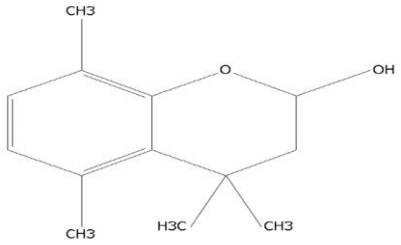
q. Ethanone, 1-(4-hydroxyphenyl)-2-phenyl-



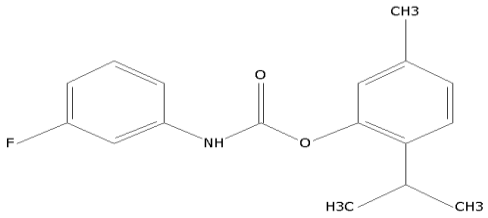
r. (1S,4S,4aS)-1-Isopropyl-4,7-dimethyl-1,2,3,4,4a,5-hexahydronaphthalene



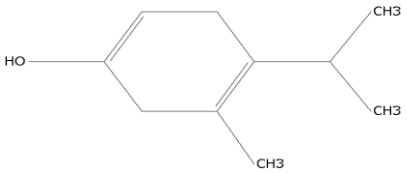
s. 4,4,5,8-Tetramethylchroman-2-ol



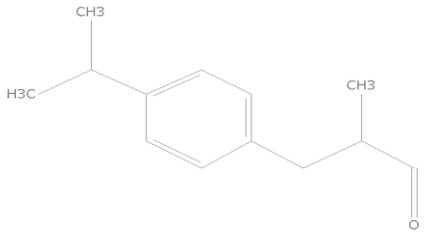
t. (3-Fluorophenyl) carbamic acid, 2-isopropyl-5-methylphenyl ester



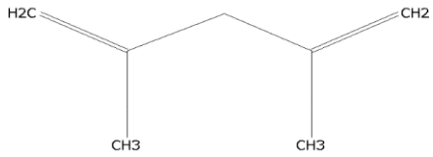
u. 3-Methyl-4-isopropylphenol



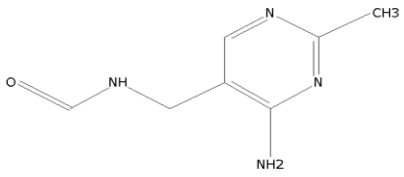
v. 3-(4-Isopropylphenyl)-2-methylpropionaldehyde



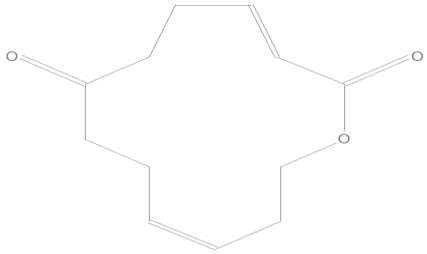
w. 2,4-Dimethyl 1,4-pentadiene



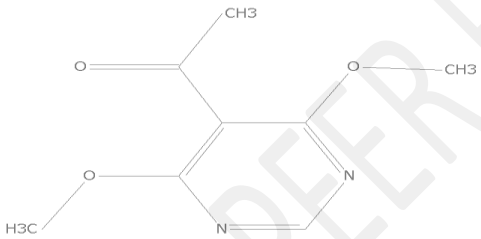
x. 4-Amino-5-formamidomethyl-2-methylpyrimidine



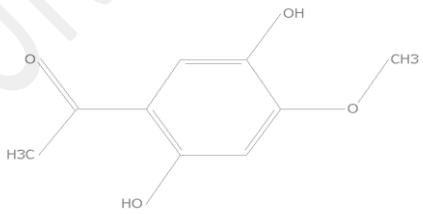
y. (3E,10Z)-Oxacyclotrideca-3,10-diene-2,7-dione



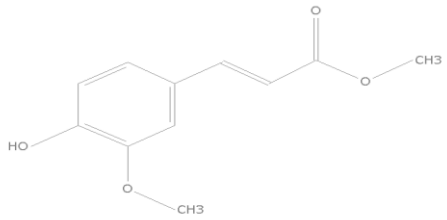
z. Pyrimidine, 4,6-dimethoxy-5-acetyl-



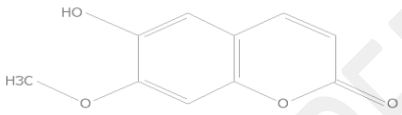
aa. 2,5-Dihydroxy-4-methoxyacetophenone



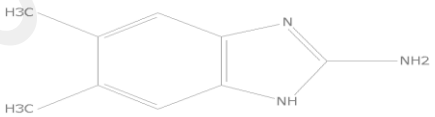
bb. 2-Propenoic acid, 3-(4-hydroxy-3-methoxyphenyl)-, methyl ester



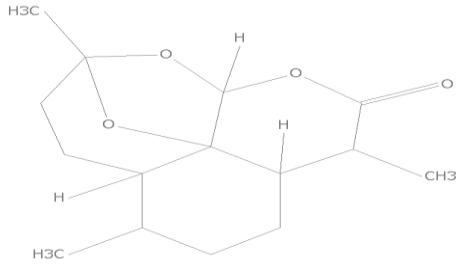
cc. 6-Hydroxy-7-methoxycoumarin



dd. 2-Amino-5,6-dimethylbenzimidazole

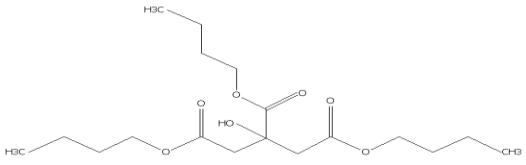


ee. Deoxyartemisinin

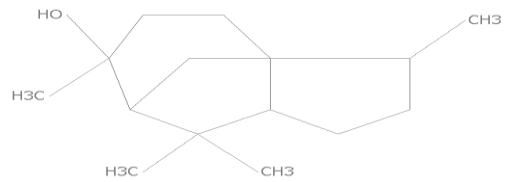


ff. Cyclohexane, 1-methyl-4-(1-methylethenyl)-, cis-

gg. Butyl citrate

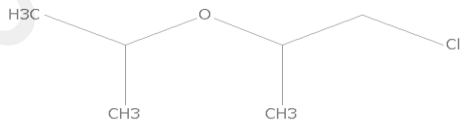


hh. Cedrol



ii. Dihydroartemisinin, 3-desoxy-

jj. Ether, 2-chloro-1-methylethyl isopropyl



kk. 1,4-Benzenedicarboxylic acid, bis (2-ethylhexyl) ester

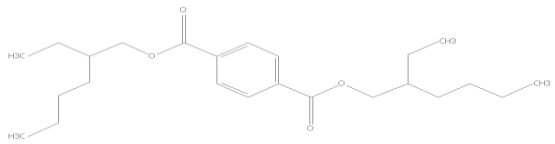


***Plate 1 a-kk: indicate 37 compounds detected in the aqeous extract of artavol® as determined by the GC-MS analysis*.**