# Benefits of Soleris® over the Conventional Method for Enumeration of Microbial Load in *Salacia* Herbal Extract

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### A b s t r a c t

Stems and roots of *Salacia* genus plants have been used as a specific remedy for early-stage diabetes, and one of the four sulphonium sulphates, salacinol is the compound responsible for the anti-diabetic activity. *Salacia* is prone to microbial contamination and insect infestation; hence, methods to estimate the microbial load in such plants will enhance its nutritional value. This paper highlights the novel use of Soleris® to quantify microbes of all types, namely bacteria, yeasts, molds, and coliforms in herbal extracts. The microbial analysis results obtained with Soleris® test vial have been compared with the conventional method, and the results indicate that Soleris® is equally efficient as the conventional method and in fact displays several advantages over the traditional method. The Soleris® method is a real time monitoring system that is highly sensitive, user-friendly, and environmentally friendly since it generates very little biomedical waste and saves a large amount of time. The data presented here demonstrate that for highly contaminated samples, results are available within 24 h. For yeasts and molds, the Soleris® method produces results in 48 h, thus offering considerable time savings compared to other commonly used methods.

K e y   w o r d s: *Salacia*, salacinol, microbial load, bacteria, yeasts, herbals

## Introduction

*Salacia*, a genus is one of the medicinal plants group broadly used to treat many ailments viz. hypoglycemia, hypolipidemia, inflammation, and diabetic patients. *Salacia* is represented by 21 species in India, of which 15 species occur in Peninsular India with *S. reticulata* and *S. oblonga* as predominant species (Bagnazari et al. 2017). The root of *Salacia* spp. (*S. chinensis*, *S. reticulata*, *S. oblonga*) is one of the preferred drug sources for treating diabetes in the indigenous systems of medicine. *Salacia* species contain two active compounds salacinol and kotalanol, which are reported to exhibit α-glucosidase inhibitory activity (Akaki et al. 2014). Apart from its anti-diabetic potential, Homma and co-workers (2019) have attributed skin whitening capabilities to salacinol with low cytotoxicity.

A wide range of diseases affecting humans has been treated since ancient times with medicinal plants, as extracts, individually or as polyherbal preparations. A survey by the World Health Organization (WHO) has indicated that in the developing countries, nearly 70–80% of the population prefers to use non-conventional herbal medicines for their primary health care (Abba et al. 2009). The herbal medicines are safe, natural, relatively accessible, and cheaper than the synthetic drugs.

Factors that influence the contamination of medicinal herbs are several. These include certain environmental factors, mainly humidity, rainfall, storage conditions of crude and processed medicinal-plant materials, and handling and hygiene of people handling the herbs. Also, microorganisms derived from soil, air and water may contaminate the herbal raw materials leading to pathogenic effects to humans (Alonzo et al. 1994; de Freitas Araújo and Bauab 2012). The presence of such pathogens limits the use of medicinal plants and also exerts an important impact on the overall therapeutic

quality of herbal drugs and preparations. Also, since the presence of such pathogenic microorganisms constitutes a potential hazard to human health, reducing their concentration from their source is critical.

*Escherichia coli* and *Pseudomonas* spp. are the typical pathogens seen in herbal extract powders. Fungal species such as *Rhizhopus, Penicillium, Aspergillus* are also reported (Hitokito et al. 1978; Kneifel et al. 2002). Although bacterial endospores and fungal spores are considered as the two dominating groups of contaminants seen on medicinal plants, the presence of pathogenic bacteria like *B. cereus, Aeromonas hydrophila, Shigella* spp., *Enterobacter agglomerans, E. cloacae, Vibrio fluvialis, Pasteurella multocida, S. epidermidis, Acinetobacter iwoffii, Klebsiell*a spp., and *B. subtilis* have been reported in plant samples analyzed recently (Idu et al. 2011).

Assessment of medicinal plants' microbial load such as *Matricaria chamomilla, Achillea millefolium, Ocimum basilicum, Calendula officinalis*, and *Tilia cordata, Hypericum perforatum*, and *Salacia* has been reported (Kumar et al. 2015; Oprea et al. 2015).

The most widely used technique for a total count of microorganisms in plant materials is a technique recommended by the WHO. In this methodology, 10 g of sample is recommended to be suspended in 90 ml of buffer sodium chloride-peptone of pH to 7.0. Suitable dilutions of the sample is plated on casein-soybean digest agar and incubated at 30–35°C. The total aerobic count is measured after 48 h. For yeast and molds, the technique employed is the sowing depth in Sabouraud dextrose plus a solution of 10% tartaric acid to obtain pH 3.0 to 3.5 and incubated at 20–25°C for five days (WHO 1998). The specification of the WHO for total aerobic microorganisms is not more than $10^7$ CFU/g, while the specification of the WHO for yeasts and molds is at most $10^4$ CFU/g. Both the Brazilian Pharmacopeia (ANVISA 2010) and the United States Pharmacopeia (USP 2005) have recommended specifications for oral use: 104 aerobic bacteria/g or ml and 102 fungi/g. The European Pharmacopoeia (EDQM 2007) also gives guidance on acceptance criteria (5.1.8 for herbal medicinal products for oral use and 5.1.4 for other herbal medicinal products). The limits of microbial contamination given in European Pharmacopoeia for herbal medicinal products to which boiling water is added before use are total aerobic bacteria ($10^7$ CFU/g), fungi ($10^5$ CFU/g) as against $10^5$ CFU/g), fungi ($10^3$ CFU) when water is not added before use.

In Ghana, around 65% of the population depends on herbal medicine and hence estimation of the accurate microbial load becomes essential in such cases to avoid health issues to herbal users (Agyeman-Duah et al. 2017). Herbal medicines in liquid form also have also been shown to have microbial contamination, and

hence determination of quantitation of microbial load becomes critical (de Sousa Lima et al. 2020). In this paper, we describe the ease of detecting microbial load in *Salacia* extract powder using Soleris® instrument and strongly believe the applicability of this method to all herbal extracts.

## Experimental

### Materials and Methods

**Reagents and chemicals**. All HPLC grade solvents (acetonitrile, methanol, water, and o-phosphoric acid) were purchased from Rankem (Bangalore, India). Standard salacinol was procured from Clear Synth, Mumbai, India.

**Collection of samples.** Stem and roots of *S. reticulata* were collected from different regions of India. The identity was confirmed and documented by Dr. P. Santhan, a taxonomist at Durva Herbal Centre, Chennai, Tamilnadu, India. The freshly collected samples of stem and roots were stored at room temperature, protected from light and humidity before analysis. Commercial samples of *S. reticulata* raw material were collected from the production unit, SAVA Healthcare, Malur, Karnataka, India.

**Preparation of aqueous extract of *Salacia* roots.** 100 g *S. reticulata* roots were pulverized and extracted with four volumes of demineralized water three times (each extraction for 3 h) at 80°C. All the three extractable liquids were later pooled and concentrated on a BUCHI rotary evaporator at 55–60°C to obtain a dry powder.

**Microbial analysis by the conventional method.** Microbiology testing of total viable count (TVC), coliform count, yeast, and mold count were carried out using standard reported methods (IS 5401-1 2012; USP 2014).

**Sterilization of *Salacia* extract.** Aqueous *Salacia* extract, as prepared above, were processed at Microtrol Sterilization Services Pvt. Ltd., Bangalore, India, for sterilization by three different methods. For steam sterilization, the *Salacia* extract was exposed to 121°C for 20 min, and the sample obtained was collected and designated as RDP/SR/070/SS01 for further analysis. For ETO treatment, the sample was exposed to a mixture of ethylene oxide and air compatible with the chamber design and introduced into the chamber at a concentration of ethylene oxide not to exceed 750 g/m³, with a dwell time of 6 h. This sample was labeled as RDP/SR/070/ES02. For gamma irradiation of the *Salacia* extract, the powder was subjected to gamma radiation to a target dose of 8 kGy in paper bags for 20 min, and the labeled sample RDP/SR/070/GR03 was used for further analysis.

All the above three samples were examined for microbial analysis using Neogen's Soleris® instrument and conventional method for total viable count, yeasts and molds, and total coliforms. The untreated extract was designated as SR011903.

**Preparation of *Salacia* extract after treatment with an anti-fungal agent.** The *Salacia* extract was treated with 50 μg of an SAVA's proprietary antifungal agent (Padmanabhan and Jadhav 2020), and the treated sample was designated as RDP/SR/068 and tested for analysis of total viable count (TVC), yeasts and molds, and total coliforms.

**Preparation of *Salacia* extract after treatment with an anti-bacterial agent.** Suitable amounts of *Salacia* extract (SR011903) was taken and treated with 50 μg of an antibacterial agent (SAVA proprietary, patent pending) in a ratio of 1:20 volumes of water at room temperature for 16 h. Next day, the material was heated to 80°C and treated with 2% activated charcoal and filtered through Hyflo supercel bed (Manju Chemtech, Bangalore, India), dried using a rotary evaporator under vacuum at 50–55°C. The dried material was used for further analysis. The treated sample was designated as RDP/SR/134 and analyzed for residual microbial counts both by conventional method and Soleris® method.

**Commercially available *Salacia* extract.** A commercial sample of *Salacia* extract was purchased from Kisalaya Herbals Limited, Indore, India, and this sample (RDP/SR/136) was examined for total viable count, yeasts and molds, and total coliforms.

**Preparation of samples for Soleris®.** 10 g of the powder extract of *Salacia* was taken in a sterile container, and 90 ml of sterile peptone water was added. The solution was mixed by vigorous vortexing for 2 min, and further dilutions up to $10^{-5}$ were prepared by adding 1 ml of the diluted solution to 9 ml of sterile peptone water. For all the analysis, 1 ml of $10^{-1}$, $10^{-3}$ and $10^{-5}$ dilutions added to the respective vials, and incubated in the machine at required temperatures as recommended by the Soleris® manufacturer.

**LC-MS/MS for determination of salacinol. Standard salacinol solution.** 2 mg of salacinol reference standard was placed in a 100 ml volumetric flask and 40 ml of diluent was added. The solution was sonicated in an ultrasonicate water bath, cooled and volume made up with the diluent and mixed by inversion. Suitable dilutions of this solution were used for LC-MS studies.

***Salacia* extract sample solution preparation.** 500 mg of *S. reticulata* was extracted and transferred into a 100 ml volumetric flask. About 70 ml of diluent was added, and sonicated for 30 min, then, cooled and made up to the mark with diluent and mixed well. Further, 2 ml of this sample solution was diluted to 200 ml with diluent, mixed well and filtered through 0.2 μm filter by discarding the first few ml of filtrate and then used.

**Raw material sample solution preparation.** 1 g of powdered dried roots of *Salacia* was taken in a round bottom flask. Nearly 100 ml of diluent (methanol) was added, and the contents were refluxed at 50–60°C for 60 min. The liquid was decanted in a clean rotary flask. The sample's refluxing was repeated by adding another 100 ml of diluent, and the collected liquids were passed through the Whatman No. 1 filter paper. The residue was washed two times with 50 ml quantity of the diluent, concentrated to ~ 50 ml by using a rotary evaporator. This concentrated liquid was diluted to 100 ml with diluent and mixed well. Further 2 ml of this solution was pipetted out in a volumetric flask and volume made up with the diluent. The solution was filtered through a 0.2 μm syringe filter for further use.

**LC-MS/MS analytical method and instrumentations.** The LC-MS method described by Akaki et al. (2014) was employed with a few modifications for better peak resolutions and separation. The LC-MS analyses were performed using a two-component system composed of mobile phase A (10% ACN/0.1% FA in water) and mobile phase B (0.1% FA in 100% ACN) at a flow rate of 0.4 ml/min. The LC-MS 8045 system (Shimadzu Co., Ltd.) was composed of an autosampler (SIL-30AC), a solvent delivery pump (LC-30AD), and a column oven (CTO-30A) with an API5000 triple-quadrupole instrument (Applied Biosystems, Foster City, CA). The Asahipak NH2P50 2D column (Shodex, Japan) with a dimension of 2 mm ID × 150 mm was used. The optimized interface parameters for the MS were with the nebulizing gas flow: 3 ml/min; heating gas flow: 10 l/min; Dl temperature of 250°C; interface temperature: 300°C and drying gas flow: 10 l/min. The mass spectrometer was operated in a multiple reaction monitoring (MRM) mode that selected one precursor ion and one suitable product ion for each target compound. The flow rate was kept at 0.3 ml/min, and the column temperature was 40°C. 5 μl of sample and standard was injected into the system, and run time was kept as 10 min. The parameters of the $m/z$ and collision energy of parent ions and quantitative product ions are shown as below:

– precursor ion: 333.4, 333.4, 333.4
– product ion: 97.0, 183.15, 231.05
– Q1 pre bias voltage: 17.0, 17.0, 17.0
– collision energy (V): 35.0, 21.0, 23.0
– Q3 pre bias voltage: 16.0, 18.0, 22.0.

## Results and Discussion

The Soleris® system consists of an incubator, ready-to-use vials, and system software that are 21 CFR complaints. The system's flexibility is appreciable since 1 to 512 samples can be tested simultaneously, and up
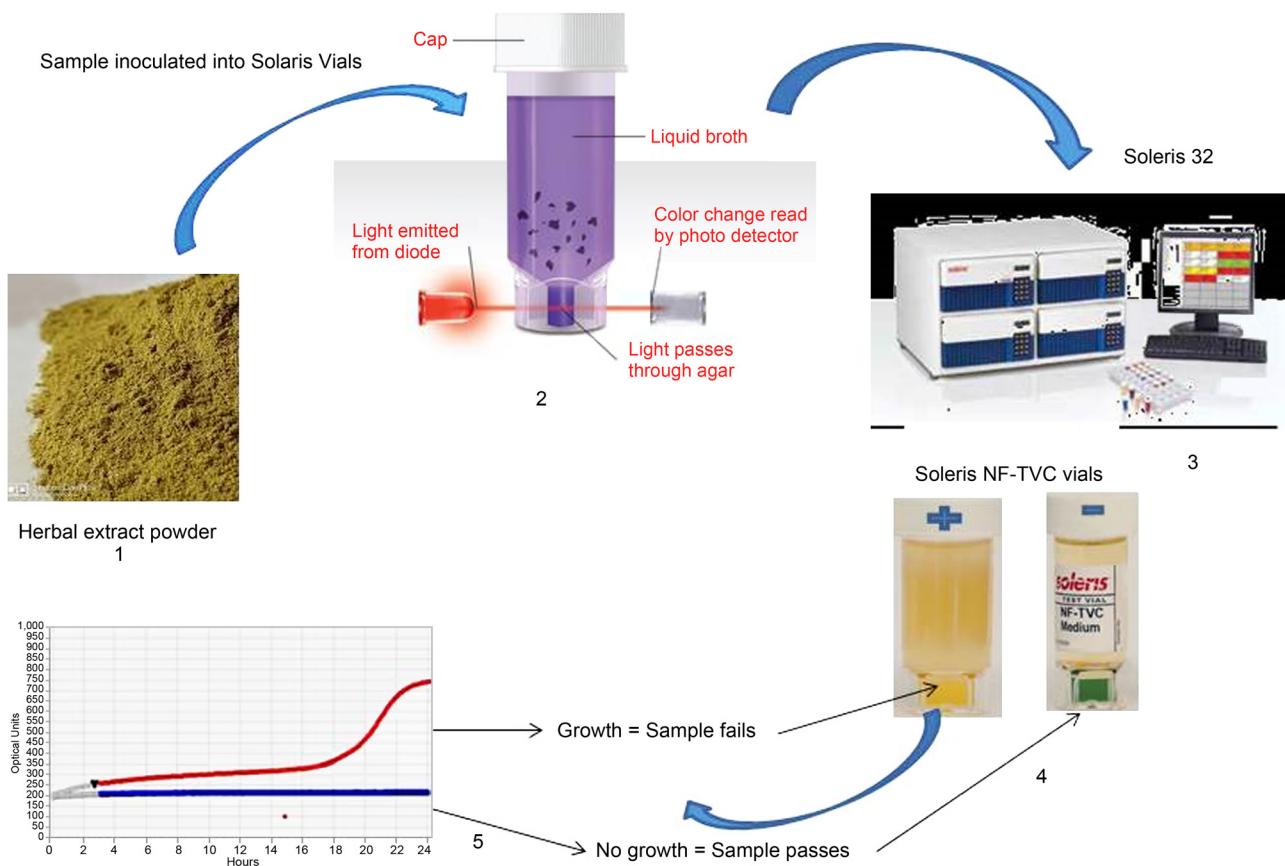
Fig. 1. Schematic representation of the Soleris® system. In Step 1, an aliquot (10 g) of the herbal extract powder is weighed in a sterile container and re-suspended in 90 ml of sterile peptone water. The solution was mixed by vigorous vortexing for 2 min and further dilutions up to $10^{-3}$ were prepared by adding 1 ml of the diluted solution to 9 ml of sterile peptone and 1 ml of the dilutions were placed in the Soleris® vials in a laminar biosafety hood under sterile conditions (Step 2). In Step 3, the inoculated vials are placed in the selected drawer location in Soleris® instrument and the vials incubated at an appropriate temperature based on type of assay chosen (TVC, yeast and molds or coliforms). As organisms grow in the broth medium, the carbon dioxide ($CO_2$) produced diffuses through a membrane layer into a soft agar plug containing a dye indicator and the change in the color of the dye is read by the Soleris® instrument (Step 4). A detection curve is generated in real time (Step 5) and the Soleris® software indicates a positive test result in less than 24 h for a sample with microbial contamination and samples producing no detection curve within 24 h are considered negative at the test threshold selected.

to four instruments can be connected to a single PC, allowing any combination of 32 and 128 sample units. The Soleris® instrument with a temperature-controlled incubator is equipped with a photodiode-based optical detection system that allows the growth of microorganisms when inoculated in incubation vials containing selective growth media, supplements, and substrates specific to the microbial species to be detected. The carbon dioxide produced metabolically by the growing microbes results in a color change as metabolic processes happen and it causes a change in pH and denotes a positive detection time (DT). The samples with a higher level of microbial contamination would show a faster DT, and hence this instrument gives a correlation between microbes present and DTs reliably.

The inoculated Soleris® vials are placed into the selected drawer location and experiments are initiated. The Soleris® software indicated positive test results in less than 24 hrs. Determinations producing no detection within 24 h were considered negative. In the case

of positive results, the growth curves were evaluated, and the visual validation of medium color change was also carried out Fig. 1. In the case of positive results, confirmation was done using conventional methods. For yeast and mold, the completed system results are available after 72 h, although positive samples were indicated after three rises in $CO_2$ levels at the preset threshold, usually within 48 h, whereas the yeast and mold detection plates required an incubation of four days and five or more days respectively.

The sensor in the Soleris® Non-Fermenting-Total Viable Count (NF-TVC) vial system utilizes detection of carbon dioxide, a universal bacterial metabolite, rather than detection of acid production, expanding the inclusivity of the vial to include non-fermenting organisms with a sensitivity of 1 CFU (Alles et al. 2009; Mozola et al. 2013). Optical readings from Soleris® test vials are graphed with time (h) in X-axis vs. optical units in the Y-axis. Table I gives the values of total viable count achieved through Soleris® and its compari-

Table I
Soleris® results for total aerobic microbial count.

| S.N. | Sample ID | Dilutions inoculated | Growth/ No growth | Detection time (h) |
|---|---|---|---|---|
| 1 | SR011903 | $10^3$ | Growth | 2.7 |
| | | $10^5$ | Growth | 4.8 |
| 2 | RDP/SR/070/SS01 | $10^2$ | Growth | 11.4 |
| | | $10^3$ | No growth | ND |
| 3 | RDP/SR/070/ES02 | $10^2$ | Growth | 12.2 |
| | | $10^3$ | Growth | 11.9 |
| 4 | RDP/SR/070/GR03 | $10^2$ | Growth | 15.1 |
| | | $10^3$ | Growth | 19.9 |
| 5 | RDP/SR/068 | $10^2$ | Growth | 10.6 |
| | | $10^3$ | Growth | 12.3 |
| 6 | RDP/SR/134 | $10^3$ | No growth | No detection |
| | | $10^5$ | No growth | No detection |
| 7 | RDP/SR/136 | $10^2$ | Growth | 13.1 |
| | | $10^3$ | No growth | No detection |

SR011903 is the *Salacia* extract batch that served as a control. RDP/SR/070/SS01, RDP/SR/070/ES02, and RDP/SR/070/GR03 refer to SR11903 treated after sterilization using steam, ETO, and Gamma irradiation respectively. RDP/SR/068 refers to batch of *Salacia reticulata* is the material treated with the anti-fungal agent that did not show any effect on the bacterial content. RDP/SR/134 and RDP/SR/136 denotes the batch taken after treatment with an anti-bacterial agent and a commercial extract of *S. reticulata* procured from a local Indian vendor.

Table II
Comparison of a total aerobic count between Soleris® (NF-TVC) and the conventional method (Salacinol content).

| S.N. | Sample ID | Total aerobic count by Soleris® (CFU/g)* | Total aerobic count by plating in Soleris® medium (CFU/g)** | Conventional plating method*** | RLOD# | Salacinol content (%) |
|---|---|---|---|---|---|---|
| 1 | SR011903 (Control) | >100,000 | 109,000 | >$10^5$ | 1.09 | 0.52 |
| 2 | RDP/SR/070/SS01 (Steam sterilized SR011903) | <1,000 | 490 | 350 | 1.40 | ND |
| 3 | RDP/SR/070/ES02 (ETO treated SR011903) | >1,000 | 1,100 | 2,700 | 0.41 | ND |
| 4 | RDP/SR/070/GR03 (Gamma irradiated SR011903) | <1,000 | 280 | 890 | 0.31 | ND |
| 5 | RDP/SR/068 (anti-fungal agent treated SR011903) | >1,000 | 1,600 | 1,500 | 1.06 | 0.506 |
| 6 | RDP/SR/134 (anti-bacterial agent treated SR011903) | >10 | 10 | 20 | 0.50 | 0.48 |
| 7 | RDP/SR/136 (commercial *Salacia* extract) | >1,000 | 1,500 | 2,300 | 0.65 | 0.059 |

ND – not done
* – time of detection <24 h
** – counts by plating the contents used for the Soleris® vials, and counts obtained after 48 h incubation
*** – time of detection ~48 h
# – relative limit of detection

son with the bacterial CFU enumerated by conventional method plating.

Fig. 2a shows the presence of bacteria for all dilutions. It is inferred through the observation of the bacterial growth curve above the baseline with the count of ~ $1 \times 10^3$ CFU/g, a value that corresponds well with the reports of Kumar et al. (2015). The dilution which does not detect any microbes shows a curve touching the baseline while a blue curve indicates the dilutions where bacteria is detected. On the other hand, the red curve indicates the highest amount of organ-

isms present in that particular or lowest dilution. The conventional method for the SR011903 sample yielded $1.09 \times 105$ CFU/g, as evident from Table II. The conventional method for the SR011903 sample yielded $1.09 \times 10^5$ CFU/g as evident from Table II. The microbial count of the *Salacia* extract after steam sterilization (SS), ETO sterilization (ET), and sterilization by gamma irradiation (GR) showed 50%, 0%, and 75% reduction respectively in comparison to the untreated control, and the values matched well when estimated by the conventional method (Table II). The CFU ranges

indicated in Table II were estimated using the Soleris® system and from the traditional plate counts done in parallel. To compare the sensitivity of the Soleris® method over the conventional method, we opted for calculation of the relative level of detection (RLOD) between the two methods as described (Mărgăritescu and Wilrich 2013). If the RLOD value was below 1, it indicated that the Soleris® method was more sensitive than the conventional plating method while the value was between 1 and 1.5, indicated both the methods to have a similar sensitivity. The RLOD value of above 1.5 indicated that the plating method was more sensitive. It is clear from Table II that the RLOD values of Soleris® in almost all the cases were sensitive over the traditional method of estimation by plating. The growth curve of sample after SS, ET and GR is represented as Figs. 2b, 2c, and 2d, respectively.

The *Salacia*'s microbial load is known to increase upon storage (Kumar et al. 2015), and gamma irradiation has been reported to reduce the microbial load by almost 2 logs. Similar results have been shown by Gupta et al. (2011) for plants such as *Terminalia chebula*, *Curcuma longa*, *Syzygium aromaticum*, and *Mentha piperita*.
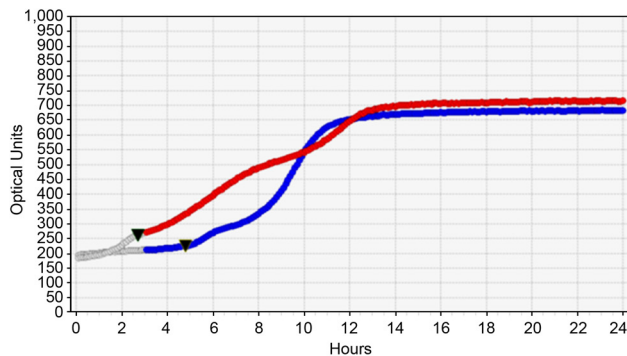
Our results shown in Table II do indicate differences in efficiencies of microbial killing for the *Salacia* extract with different agents. While gamma irradiation and steam sterilization showed promising microbial load reduction, the ETO treatment did not show any reduction in the microbial load. This could be attributed to the type of microorganism present in *Salacia* extract and the insufficient ETO dose required to efficiently kill resident microbes. Interestingly, differences in efficiency of killing of microorganisms contaminating the herbal extracts has been shown to depend on the types of contaminating organisms, their chemical and physical structure, antiseptic properties of different plants, and their ability to recover from the radiation injury (Gupta et al. 2011), hence, the dose required for efficient microbial killing could be different for different plants.

It is evident from the current observations that none of the traditional methods are efficient enough to reduce the microbial load in *Salacia,* and hence, our observations of reductions in microbial counts in sample RDP/SR/134 by almost 2 logs through the use of an antibacterial agent (proprietary, patent-pending)



Fig. 2. a) Microbial growth curve in Soleris® NF-TVC vials of various dilutions. Red curve denotes growth with sample SR011903 of $10^{-3}$ dilution while blue curve denotes $10^{-5}$ dilution of the sample. Microbial detection was seen in both the dilutions.



Fig. 2. c) Sample RDP/SR/070/ES02 showed detection of microbes in all the dilutions in Soleris® NF-TVC vials. Blue curve denotes $10^{-3}$ dilution while the $10^{-2}$ dilution is represented by the red curve.
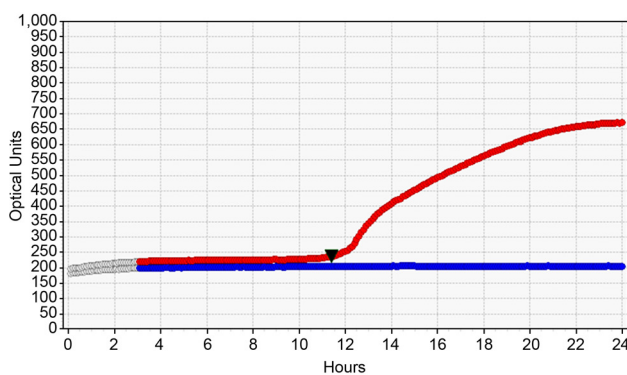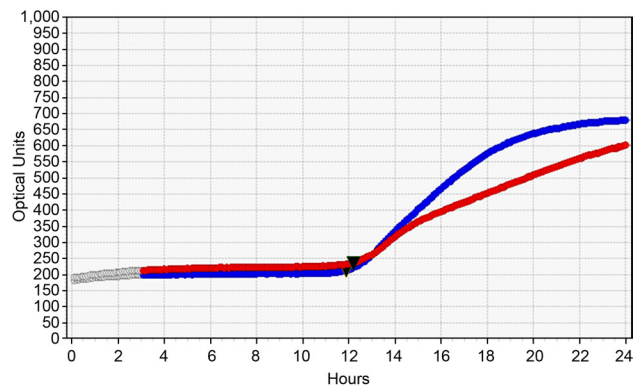


Fig. 2. b) Sample RDP/SR/070/SS01 showed positive results for NF-TVC in Soleris® at $10^{-2}$ dilution (red curve) while the blue curve denotes $10^{-3}$ dilution.
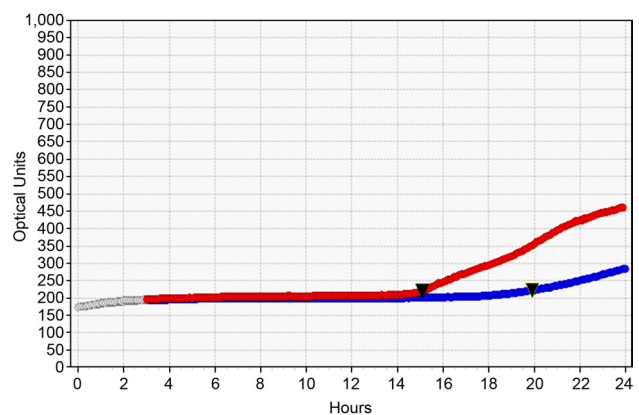


Fig. 2. d) Sample RDP/SR/070/GR03 showed detection in all the dilutions in Soleris® NF TVC vials. Blue curve denotes $10^{-3}$ dilution while the red curve denotes $10^{-2}$ dilution.
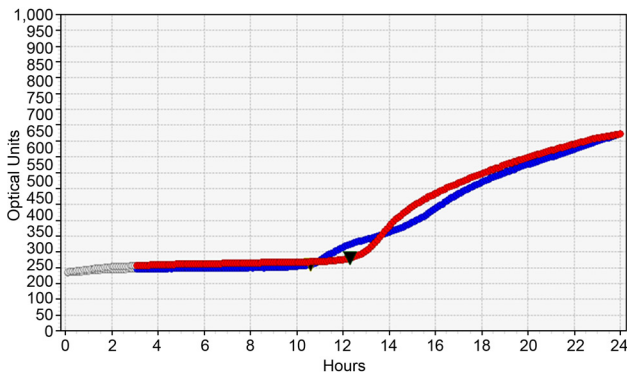
Fig. 2. e) Sample RDP/SR/068 showed detection in all the dilutions in Soleris® NF-TVC vials. Red curve denotes $10^{-3}$ dilution while the blue curve shows growth with $10^{-2}$ dilution of the sample.
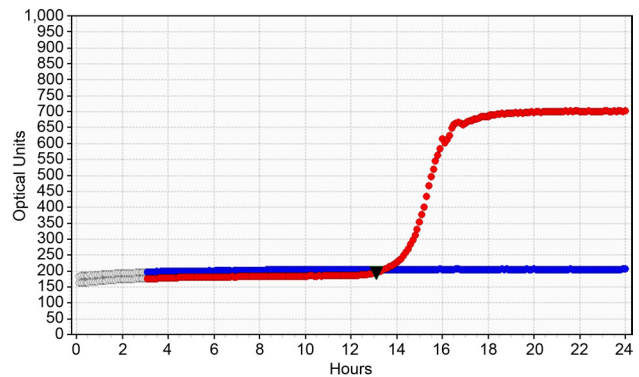


Fig. 2. g) RDP/SR/136 sample showed microbial detection only in $10^{-3}$ dilution in NF-TVC Soleris® vials denoted by the red curve. The blue flat line denotes no growth seen with $10^{-5}$ dilution of this sample.
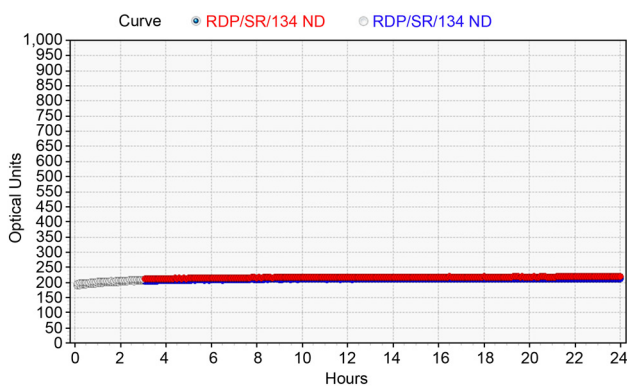


Fig. 2. f) RDP/SR/134 sample showed no bacterial growth in any of the dilutions ($10^{-3}$ and $10^{-5}$) tested. This is represented by blue and black flat lines respectively.

appear promising and can be taken up for trials in manufacturing scales.

The sterilization methods using radiations are not readily adaptable for manufacturing plant processes since it requires special equipment and methods. The other methods, such as ethylene oxide, are banned in European Union countries due to the generation of carcinogenic substances such as ethylene glycol, 2-chloroethanol. Also, steam sterilization is not often used for herbal materials since the treated materials become clumped after steam treatment (Brodowska et al. 2014). A new alternative Electron-beam (E-beam) technology, is being used in the food industry to decontaminate food materials (Silindir and Özer 2009). Since installations of such specialized equipment would require investments, alternative methods that are cost-effective to sterilize materials, such as what we describe here, appear to be an attractive proposition.

It is also clear from Table II that the commercially available *Salacia* extract (sample ID RDP/SR/136) showed a TVC of > 1,000 CFU/g. The growth curve seen for TVC for samples RDP/SR/068, RDP/SR/134, and RDP/SR/136 is shown in Figs. 2e, 2f and 2g, respectively. Sample RDP/SR/068 showed microbial detection

in all the dilutions ($10^{-3}$ and $10^{-2}$) which accounts for ~ 1,600 CFU/g obtained by plating one of the dilutions while sample RDP/SR/134 showed no bacterial growth in any of the dilutions ($10^{-3}$ and $10^{-5}$) tested, and showed merely 20 CFU/g from $10^{-1}$ dilution reflecting the efficacy of the antibacterial agent in lysing the microbes contaminating the Salacia extract. RDP/SR/136 sample showed nearly 1,500 CFU/g as seen with the $10^{-2}$ dilution in NF-TVC Soleris® vials denoted by the red curve (Fig. 2g).

It is evident from Table III that the Soleris® can detect yeast and molds when present within 24 h while it takes more than 5 days when tested by conventional methods. It is also clear that when the extract was pre-treated with the proprietary antifungal agent, the material (RDP/SR/068) showed > 99% reduction in the count of yeasts and molds (Table IV). The Soleris® Direct Yeast and Mold (DYM 109-C) method offers huge time savings for labs engaged in microbial identification in herbal powders. While the yeast and mold results are accessible within 48 h by Soleris®, the conventional methods take up to 5 days. None of the samples tested here showed a growth curve for yeast and molds with the least dilutions except for SR011903 and RDP/SR/136, represented by Figs. 3a and 3b, respectively. Supplementary figures represent the growth curve of other samples, namely SR/011903, RDP/SR/070/SS01, RDP/SR/070/ES02, RDP/SR/070/GR03, RDP/SR/068 and RDP/SR/134, Figs. S1a, S1b, S1c, S1d, S1e, and S1f, respectively. It is interesting to emphasize here that the total yeast and mold counts of the *Salacia* extract enumerated using Soleris® and conventional method of ~ 290 to 380 CFU/g matches well with the reported values of yeast and molds in *Salacia* by other workers (Kumar et al. 2015) demonstrating the sensitivity and accuracy of the Soleris® method of microbial enumeration in the herbal extract of *Salacia* as tested here.

As far as the total coliforms counts were concerned, we found similar results of Solaris® against the
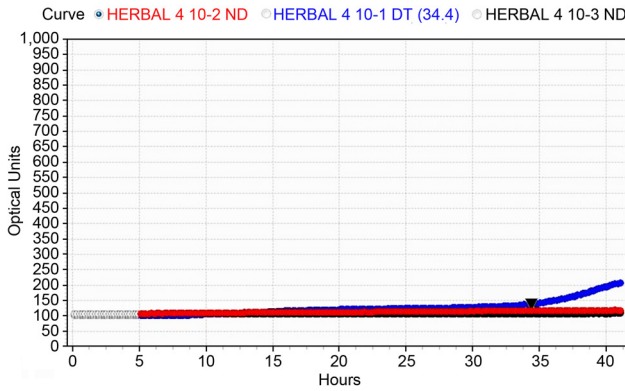
Fig. 3. a) Sample SR011903 showed microbial detection in 10⁻¹ dilution in Soleris® DYM 109-C vials represented by blue curve. Black curve denotes 10⁻³ dilution while the red curve denotes 10⁻² dilution, both of which did not show any growth.
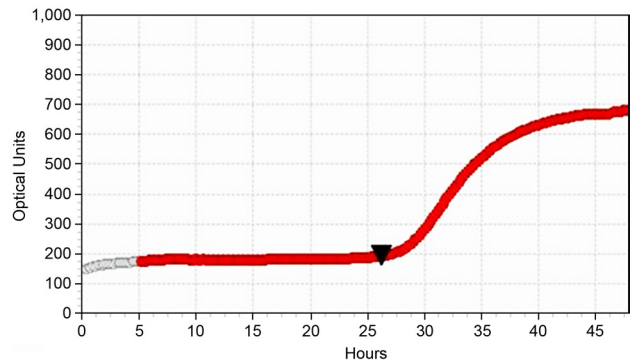


Fig. 3. b) Sample RDP/SR/136 showed microbial detection in $10^{-1}$ dilution for DYM 109-C Soleris® vials represented by the red curve.

conventional method, indicating the specificity of the tests employed. None of the samples showed any count for total coliforms both by conventional method and by Soleris® test as evident from Table SIa and SIb (supplementary tables). The growth curve pattern on Soleris® also did not show any growth-related optical unit readings (data not shown).

Health surveys conducted in several countries have demonstrated the use of herbal medicines as a mainstream practice among the elderly population, and

hence the risk of microbial contamination in herbal drugs might have an adverse effect on the health of such population (de Medeiros et al. 2013; Famewo et al. 2016). Several pathogenic bacteria have been detected in some of the herbal medicines, which is also a severe concern for such medicines' quality issues (Abba et al. 2009; de Medeiros et al. 2013). Hence, it becomes imperative to estimate microbial load in such medicinal herbs and extracts so that such material consumption is safe without any side effects.

Table III
Soleris® results for the total yeast and mold counts.

| S.N. | Sample ID | Dilutions inoculated | Growth/ No growth | Detection time (h) |
|---|---|---|---|---|
| 1 | SR011903 | $10^1$ | Growth | 34.4 |
| | | $10^2$ | No Growth | – |
| | | $10^3$ | No Growth | – |
| 2 | RDP/SR/070/SS01 (Steam sterilized SR011903) | $10^1$ | No Growth | – |
| | | $10^2$ | No Growth | – |
| | | $10^3$ | No Growth | – |
| 3 | RDP/SR/070/ES02 (ETO treated SR011903) | $10^1$ | No Growth | – |
| | | $10^2$ | No Growth | – |
| | | $10^3$ | No Growth | – |
| 4 | RDP/SR/070/GR03 (Gamma irradiated SR011903) | $10^1$ | No Growth | – |
| | | $10^2$ | No Growth | – |
| | | $10^3$ | No Growth | – |
| 5 | RDP/SR/068 (anti-fungal agent treated SR011903) | $10^1$ | No Growth | – |
| | | $10^2$ | No Growth | – |
| | | $10^3$ | No Growth | – |
| 6 | RDP/SR/134 (anti-bacterial agent treated SR011903) | $10^1$ | No Growth | – |
| | | $10^2$ | No Growth | – |
| | | $10^3$ | No Growth | – |
| 7 | RDP/SR/136 (commercial *Salacia* extract) | $10^1$ | Growth | 26.2 |
| | | $10^2$ | No growth | – |
| | | $10^3$ | No growth | – |

Table IV
Comparison between Soleris® (DYM-109) and the conventional method
for the total yeasts and molds.

| S.N. | Sample ID | Soleris® (CFU/g) | Conventional method (CFU/g) | Acceptable/Not acceptable |
|---|---|---|---|---|
| 1 | SR011903 | > 10 | 380 | Not acceptable |
| 2 | RDP/SR/070/SS01 | < 10 | < 10 | Acceptable |
| 3 | RDP/SR/070/ES02 | < 10 | < 10 | Acceptable |
| 4 | RDP/SR/070/GR03 | < 10 | < 10 | Acceptable |
| 5 | RDP/SR/068 | < 10 | < 10 | Acceptable |
| 6 | RDP/SR/134 | < 10 | < 10 | Acceptable |
| 7 | RDP/SR/136 | > 10 | 290 | Not acceptable |

SR011903 is the *Salacia* extract batch that served as a control. RDP/SR/070/SS01, RDP/SR/070/ES02 and RDP/SR/070/GR03 refer to SR11903 treated after sterilization using steam, ETO and gamma irradiation respectively. RDP/SR/068 refers to batch of *Salacia reticulata* is the material treated with the anti-fungal agent which did not show any effect on the bacterial content, RDP/SR/134 and RDP/SR/136 denote the batch taken after treatment with an antibacterial agent and a commercial batch of *S. reticulata* procured from a local market.

The killing of microorganisms contaminating the herbal extracts is dictated by the dose rate of the antimicrobial agent employed and also by the physical state of the growth of the microbial cells, and other external factors, such as oxygen, water, other chemical agents, and temperature (Halls 1992). Hence, our current data on achieving a microbial reduction of > 99% in *Salacia* is attractive, and obtaining such data from a sensitive instrument like Soleris® assumes critical importance. The Non-Fermenting Total Viable Count (NF-TVC) and the Direct Yeast and Mold (DYM) test methods have been used for various food matrixes, and these have been validated as alternatives to conventional plating techniques (Limberg et al. 2016). The advantages of these growth-based automatic measurements in contrast to conventional plate-counting methods include precision, accuracy, reproducibility, speed, and cost (Blivet 2014; Curda and Svir'akov'a 2014).

detection of microorganisms. The Soleris® test from Neogen is approved from the AOAC Research Institute as a Performance Tested Method certification 071203 for detecting microorganisms in as little as 4 h, and only takes 24 h to a negative result. The Soleris® vial is substituted for the agar plate. The sensitivity of the equipment is 1 CFU/g. The significant reduction in the generation of biomedical lab waste concerning Solaris® compared to the conventional methods is really attractive. Since Solaris® system reads the vials photometrically by looking into the color change relative to the starting color, it would be the choice of method for all researchers in academics and industries alike who are engaged in determining the microbial contamination in food and other natural products.

## Conclusions

Microbial contamination in herbal extracts is one of the issues addressed by the new FDA regulations, and faster and more streamlined microbiological tests are therefore required by the herbal industry to meet the new challenges. Soleris® TVC method has been successfully used for rapid and accurate detection of microorganisms in a variety of food commodities, and its technology is based on monitoring pH change or $CO_2$ production as a means of microbial growth activity in media vials. The performance of the Soleris® TVC method in detecting microbes in herbal extracts is the first report to date. The sensitivity of the Soleris® method was found to be comparable to that of the reference procedures for the

## Conflict of interest

The authors do not report any financial or personal connections with other persons or organizations, which might negatively affect the contents of this publication and/or claim authorship rights to this publication.

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