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MICROBIAL EVALUATION OF ALLOPATHIC AND HERBAL NON-STERILE PHARMACEUTICAL LIQUID DOSAGE FORMS

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ABSTRACT

Nonsterile pharmaceutical finished products are not manufactured through aseptic processes so are not completely free from microbial contaminations, which can impact their efficacy due to the degradation of active ingredients. The aim of present study was to enumerate, isolate and identify the microbial contaminations in non-sterile liquid finished pharmaceutical dosage forms. A total of 50 nonsterile liquid products were collected from different pharmacies across Lahore city, Pakistan. Pour plate method was used to test the total microbial count as per USP guidelines. The results showed a microbial count of 140 cfu mL^{-1} in nutraceuticals manufactured liquid dosage form, 60 cfu mL^{-1} in multinational products, 120 cfu mL^{-1} in registered herbal products, and 390 cfu mL^{-1} in non-registered herbal liquid products. Major bacterial contaminants isolated from allopathic (local and multinational) products and herbal (registered and non-registered) products are reported.

Keywords: Non-sterile products; allopathy; herbal products; pathogenicity; microbial contamination; naturopathy

INTRODUCTION

Various dosage forms are the means for delivery of drugs within a body. The pharmaceutical dosage form is categorized as sterile and non-sterile groups. But non-sterile dosage form should be within the microbiological limits prescribed in the monographs of pharmacopoeias and such products should therapeutically be effective and safe for use (Allen and Ansel, 2013; Lee *et al*., 2022). Non-sterile pharmaceutical products are not manufactured through aseptic processes so are not free from microbes (Cundell, 2019; Jimenez *et al*., 2000). A contaminated non-sterile liquid pharmaceutical product can lose its effectiveness and become harmful for the patients (Eissa, 2016). The degree of contamination in non-sterile product should be synchronized and based on the acceptance criteria for microbiological quality established in pharmacopeia monographs (Gad *et al*., 2011; Adigwe *et al*., 2022).

The major contaminants of non-sterile pharmaceutical end products and raw materials are bacteria, yeast and moulds (Myemba *et al*., 2022). The low lipid emulsion formulations promote rapid microbial growth at room temperature (Delgado-Pando *et al*., 2011; Yazgan, 2022). Contaminants can enter the manufacturing process through various sources such as poor facility design, personnel, equipment for production and machinery (Hu *et al.*, 2016). There are three types of viable particulate contaminants: pathogens, opportunistic pathogens and non-pathogenic organisms. A pathogen is a

living organism that causes illness to its host (Jangra *et al*., 2022) while opportunistic microbes are the ones which infect under certain circumstances such as decreased immunity (Brown *et al*., 2012; Gorrie *et al*., 2022). The microbial contents of non-sterile products are very important. Excessive growth or contamination may result in immediate or delayed unwanted effects like autoimmune reactions and cancers (Agaronyan *et al*., 2022). Moreover, microbial growth can have unfavourable impact on their efficacy due to degradation of active ingredients and simultaneous production of harmful chemicals (Dou, 2022). The presence of sugar and moisture serve as nutrient for the growth and survival of microorganisms (Owusu-Apenten and Vieira, 2022). Thus, it is essential to maintain no growth for sterile products and low microbial bioburden for non-sterile drugs. Nonsterile liquid dosage forms constitute huge portion of pharmaceutical products that are available in Pakistani market. The present research is a pioneer work in this area of Pakistan. Likewise, good production, distribution, and storage techniques have not been compared between locally produced and imported goods. The present work was aimed to conduct research in this area as well as motivate other researchers to explore this important aspect of health issue.

MATERIALS AND METHODS

Sample collection

The samples were collected from different pharmacies across the Lahore city in Pakistan. The study comprised of collecting three batches of each product. Fifty non-sterile liquid dosage forms were collected. To compare the microbial quality of dosage forms, the sample of national and multinational products (allopathic) were collected. Registered and non-registered products were included for herbal medicines. The samples comprised of 15 products from each of nutraceuticals and multinational companies` medicines. Ten each registered and non-registered herbal products were also included in the study. Each product was assigned a specific product code to conceal its identity. Allopathic nutraceuticals products and allopathic multi-national products were assigned code from AL-1 to AL-15 and AM-1 to AM-5, respectively. Herbal non-registered and registered samples were assigned code from HN-1 to HN-10 while herbal registered products were assigned code from HR-1 to HR-10.

Total microbial count (TMC) test

The samples were prepared for total microbial count (TMC) test as per the procedure mentioned in the harmonized chapter <61> in US Pharmacopeia. Pour plate was used to conduct TMC test. Ethanol (70%, v/v) was used to swab the outer surfaces of all the containers before opening. Liquid product (10 mL) e ach was accurately measured and aseptically dissolved into 90 mL trypticase soy broth (TSB) medium (Merck®, Merck Millipore-Germany) and dispersed. Then, 1 mL dissolved product was aseptically taken in a petri-plate and incubated at 30-35°C for 48 h. The 20 mL molten trypticase soy agar (TSA) medium (Merck®, Merck Millipore-Germany) was added on the samples taken. The petri-plates were swirled gently, covered with lids, inverted after solidification and incubated at 30-35°C for 3 days. The above procedure was repeated using Sabouraud dextrose agar (SDA) medium (Merck®, Merck Millipore-Germany) instead of TBA which was then incubated at 20-25°C for 7 days.

Isolation of microbes

The incubated TSB was examined for the presence of *Staphylococcus aureus* by streaking on plates of mannitol salt phenol red agar (MSPRA) medium (Merck®, Merck Millipore-Germany) [72 h incubation at 35°C]. The growth was observed and isolate Gram stained. The results were confirmed by using API® staph (BioMérieux- France) (Bio-chemical differentiation test).

To isolate *Pseudomonas aeruginosa*, cetrimide agar (CA) (Merck®, Merck Millipore-Germany) medium used for streaking the plates (48 h incubation at 35°C) from incubated TSB. The resulted growth was observed, the isolate Gram stained, its fluorescence checked in UV light and oxidase test performed. The presence was confirmed by API 20 NE (Biochemical differentiation test).

To isolate *Salmonella typhi*, lactose broth (LB) medium (Merck®, Merck Millipore-Germany) was used. The product (10 mL) was aseptically added to 90 mL LB medium (broth concentrations 2 fold), dispersed and incubated for 24 h at 35°C. The contents were mixed and 1 mL poured to 100 mL enrichment medium, Enterobacter enrich broth Mossel (EBM) (Merck®, Merck Millipore-Germany). It was incubated along with the remaining LB at 35°C for 24 h. After incubation, 1 mL portions were put into the tubes containing 10 mL selenite enrichment broth (double strength), gently mixed and incubated (24 h). Sub-culturing was done on three media (two plates for each) i.e. brilliant green agar (BGA), xylose lysine deoxycholate agar (XLD) and bismuth sulfite agar (BSA) media (Merck®, Merck Millipore- Germany).

Gram staining was performed to confirm the morphology of isolated bacteria. The suspected colonies were also streaked on the triple sugar iron agar (TSI) (Merck®, Merck Millipore-Germany) slant surfaces by streaking, then piercing the wire well underneath the surface. These were incubated for 24 h at 35°C. The slants were checked for the colour change i.e. alkaline (red) and but become acidic (yellow), with simultaneous colour change to black in case of hydrogen sulphide production.

The growth present in EBM was mixed gently, sub-cultured on the plates of levine EMB (LEMB) agar medium (Merck®, Merck Millipore-Germany) and incubated for 24 h at 37°C. The resulted growth was observed microscopically, and Gram stained. The results were confirmed by using API 20 E (Biochemical differentiation test).

To isolate *Candida albicans* from a product, TSB (100 mL) was inoculated with 10 mL test product, mixed and incubated for 5 days at 35°C. After mixing well, petri-plates of SDA were sub-cultured and incubated at 35°C 48 h. The isolated colonies were Gram stained and examined microscopically.

Biochemical differentiation test (API® Staph, 20 E, 20 NE)

To conduct biochemical differentiation test API® (BioMérieux-France) strips were used. These strips provide accurate and precise identification of target microbes. These strips consist of 20 miniatures. THR-8e miniatures served as an individual biochemical test. The API® standard kit (Fig. 1) consisted of an incubation box, strip and reagents. The biochemical differentiation test was performed as per supplier instructions to identify isolated microorganisms.

Fig. 1: Biochemical differentiation test using API 20NE

RESULTS AND DISCUSSION

The microbial load in nutraceuticals non-sterile liquid dosage forms showed that the product AL-15 had a maximum number of bacteria (140 cfu mL^{-1}), whereas product AL-5 had minimum number of bacteria (10 cfu mL^{-1}). In every product the yeast contamination level was <10 cfu mL^{-1}. Five products showed microbial count above the permissible limit (i.e. $\geq$ 50 cfu mL^{-1}). These products included AL-2, AL-3, AL-7, AL-10 and AL-13. Two products AL-12 and AL-15 exceeded the total bacterial limit (i.e. $\leq$100 cfu mL^{-1}). Products AL-1 and AL-2 were contaminated with *Staphylococcus aureus* and *Sphingomonas paucimobilis*. The opportunistic pathogens *Citrobacter freundii* and *Staphylococcus xylosus* were also isolated from AL-1. Products AL-8 and AL-11 were contaminated with opportunistic pathogen *Staphylococcus warneri* whereas, *Staphylococcus lentus* was isolated from product AL-13. The opportunistic pathogens *S. warneri*, *C. freundii* and *S. lentus* were isolated from

product AL-15. Products AL-3, AL-4, AL-5, AL-6, AL-7, AL-9, AL-10, AL-12 and AL-14 were found free from pathogens and opportunistic pathogens.

In multinational liquid dosage forms, the products AM-4, AM-5, AM-6, AM-7, and AM-15 showed minimum number of bacteria (i.e. <10 cfu mL^{-1}). Maximum number of molds was observed in product AM-1 (10 cfu mL^{-1}) and minimum in products AM-2 to AM-12 and AM-15 (<10 cfu mL^{-1}). All AM series product were found free from yeast contamination. Product AM-1 showed microbial load above the alert limit (i.e. $\geq$ 50 cfu mL^{-1}). In products AM-3 and AM-10 the microbial counts were near to the alert limit. No product of liquid dosage forms was beyond total bacterial limit (i.e. $\leq$100 cfu mL^{-1}).

Multinational liquid dosage forms showed less microbial count results as compared to the nutraceuticals liquid dosage form (Fig. 2). Due to the presence of lesser microbial count, multinational liquid dosage forms were less likely to be contaminated with pathogens. The products AM-1 and AM-9 were contaminated with opportunistic pathogen *S. lentus*. Opportunistic pathogen *Pseudomonas luteola* was isolated from AM-2. Opportunistic pathogen *S. warneri* was isolated from products AM-11 and AM-13. Products AM-3, AM-4, AM-5, AM-6, AM-7, AM-8, AM-10, AM-12, AM-14 and AM-15 were free from any pathogen.

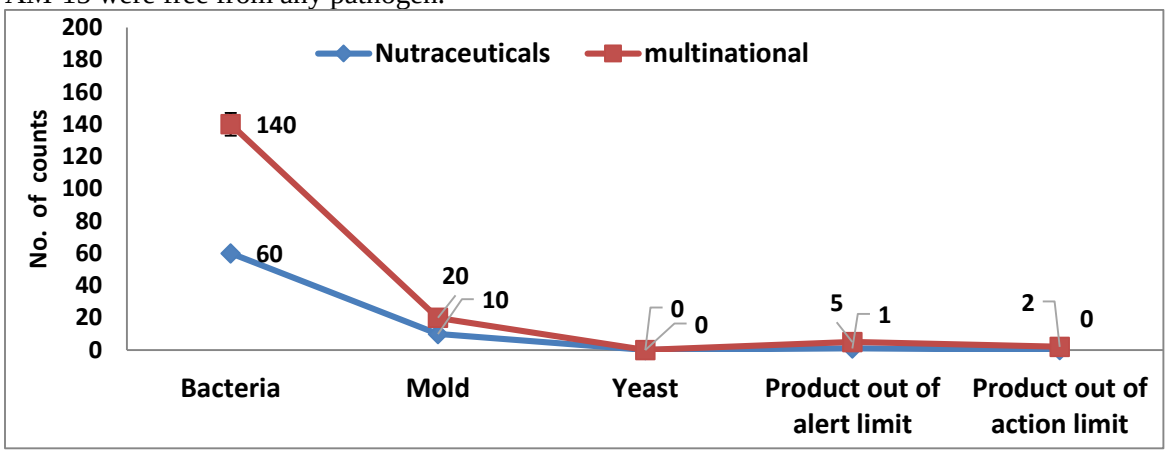

Fig. 2: Comparison of microbial load in nutraceuticals manufactured and multinational liquid dosage forms

In non-sterile liquid dosage forms of herbal origin, the product HR-2 had maximum number of bacteria (120 cfu mL^{-1}), whereas products HR-5, HR-6 and HR-8 had minimum bacterial count (10 cfu mL^{-1}). Maximum mould count was observed in product HR-3 (30 cfu mL^{-1}) and minimum in products HR-5, HR-6 and HR-7 (<10 cfu mL^{-1}). Total bacterial count of product HR-2 was out of the permissible limit ($\leq$ 100 cfu mL^{-1}), whereas 4 products exceeded this limit (i.e. $\geq$ 50 cfu mL^{-1}). These products included HR-1, HR-4, HR-7 and HR-9. The product HR-1 was contaminated with pathogen *S. paucimobilis* and opportunistic pathogen *P. luteola*. HR-2, HR-3, HR-5, HR-6, HR-8 and HR-10 were contaminated with opportunistic pathogen *Staphylococcus hominis*, *S. warneri* and. *P. luteola*. *S. lentus* was isolated from product HR-7. Opportunistic pathogens *S. lentus* and *S. xylosus* were isolated from product HR-9. Product HR-4 was found free from both pathogens and opportunistic pathogens.

Non-registered herbal liquid dosage forms due to their mal manufacturing processes are more susceptible to microbial growth. The product HN-10 had maximum bacterial count (390 cfu mL^{-1}), whereas product HN-6 had minimum count (30 cfu mL^{-1}). Maximum number of moulds was observed in products HN-1, HN-5 and HN-10 (30 cfu mL^{-1}) and minimum in product HN-6 (<10 cfu mL^{-1}). Maximum yeast count was seen in products HN-7 and HN-9 (20 cfu mL^{-1}) and minimum in products HN-1, HN-3, HN-4 and HN-8 (i.e. <10 cfu mL^{-1}). Bacterial counts in 8 products out of 10 crossed permissible limit (i.e. < 100 cfu mL^{-1}). These products included HN-1, HN-2, HN-3, HN-5, HN-7, HN-8, HN9 and HN-10. One product HN-4 showed microbial counts in alert limit (i.e. $\leq$ 50 cfu mL^{-1}).

Non-registered herbal liquid dosage forms are not manufactured as per procedures set by international pharmaceutical standards. The Fig. 3 presents a comparison between registered and non-

registered products. The products HN-4 and HN-9 were contaminated with pathogen *S. paucimobilis*. Opportunistic pathogen *S. warneri* was isolated from HN-1, HN-2, HN-7 and HN-10. *P. luteola* was isolated from products HN-3, HN-6 and HN-9. Opportunistic pathogens *C. freundii* was present in HN-2, HN-5 and HN-7. *S. lentus* was isolated from products HN-1, HN-3, HN-6 and HN-7. Product HN-4 was contaminated with *S. xylosus*. *S. hominis* was present in HN-5 and HN-9.

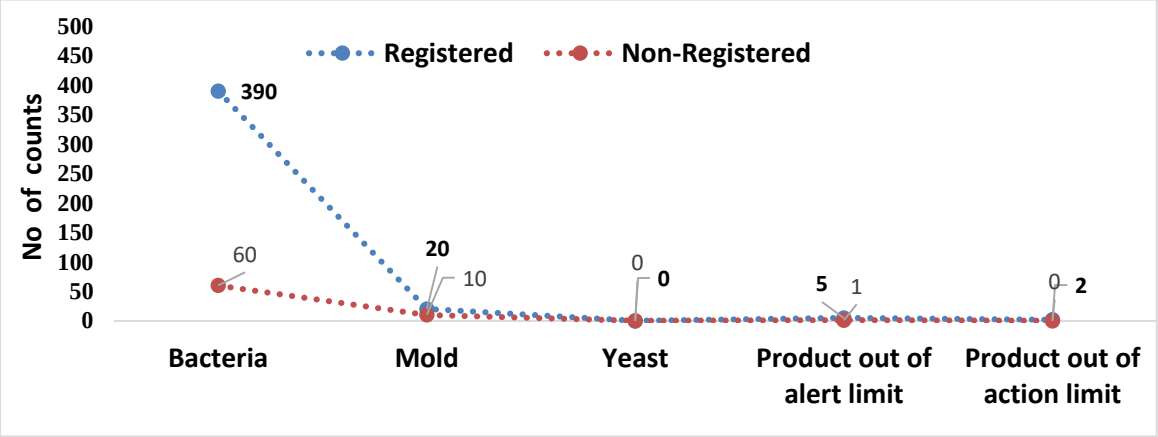

Fig. 3: Comparison of microbial load in registered and non-registered herbal liquid dosage forms

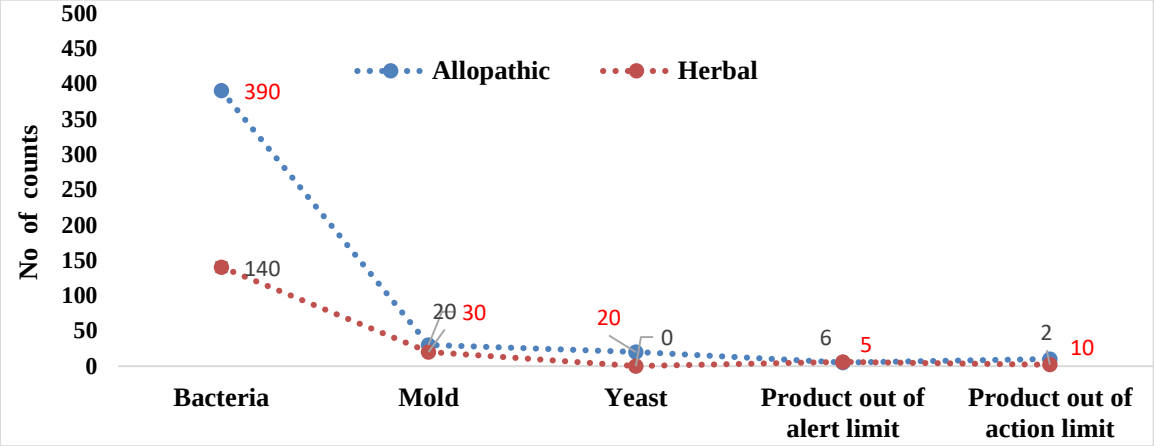

Fig. 3: Comparison of microbial load in allopathic and herbal liquid dosage forms

Fig. 3 shows a comparison of microbial load in herbal and allopathic liquid dosage forms, with maximum bacterial load in herbal liquid dosage form (390 cfu mL^{-1}) as compared to the maximum load of 140 cfu mL^{-1} in allopathic liquid dosage forms. Allopathic liquid dosage form contained 20 cfu mL^{-1} of moulds and herbal liquid dosage form contained 30 cfu mL^{-1}. Herbal liquid dosage forms contained more recovered moulds than allopathic liquid dosage form. In case of yeast recovery both herbal and allopathic liquid dosage form showed better results. Maximum yeast in allopathic and herbal liquid dosage forms was <10 and 20 cfu mL^{-1}, respectively.

Liquid dosage forms are more prone to microbial contamination. All the raw materials which can facilitate the microbial growth of liquid dosage form should undergo microbial limit test before their use (Folashade *et al*., 2012; Khana *et al*., 2018). Controlled environment and validated manufacturing procedures play key role in controlling microbial contamination during processing/manufacture (Khana *et al*., 2018). In present study bacterial count exceeded in a few products tested, indicating possibility of contamination, especially those of opportunistic pathogens. It is important to understand that during shelf life of a product, the bacterial count is expected to increase further. Good preservatives can help to control such growths. Products showing higher microbial count also reflect poor environmental control and possible contamination of raw materials and packaging materials.

S. aureus lives on the skin and mucous membrane of humans (Hussain *et al*., 2021; Myemba *et al*., 2022). Product AL-1 was contaminated with this pathogen which reflects poor personal handling during manufacturing process. In addition, poor equipment cleaning and improper gowning of the personal contribute towards this contamination. *S. paucimobilis* was isolated from product AL-2. It usually comes from contaminated distilled water use for manufacturing (Salem *et al*., 2021). Improper water quality check in pharmaceutical product is the possible cause of this contamination. *C. freundii* was isolated from product AL-1. Its habitat includes environment (soil, water, sewage) and human intestinal tract. All liquid dosage forms containing water in higher proportions are at risk in contaminated with this opportunistic pathogen. *S. xylosus* was isolated from product AL-1. This bacterium is normally present on human and animal skin (Cundell *et al*., 2019). Contamination with this opportunistic pathogen could be due to poor personal handling during manufacturing process, poor equipment cleaning procedure and improper gowning. *S. lentus* contamination comes from food producing animals and their handlers (Eissa *et al*., 2016). Raw materials of animal origin may be the possible cause of products contaminated with this opportunistic pathogen. Isolation of *S. lentus* from products AL-13 and AL-15 indicates that the raw materials for these products are of animal origin.

Multinational liquid dosage forms showed better microbial quality in test products. Addition of good preservative, adopting cGMP and effective quality control testing contribute towards product quality. Microbial contamination level during product release defines its quality. Higher levels of contamination proportionally increase the chance of product degradation. Product AM-1 microbial load was found above the alert limit because of its sugar contents. Sugar facilitates the microbial growth especially moulds. In products AM-3 and AM-10 the microbial counts were near alert limit due to the presence of raw materials which facilitate microbial growth. Addition of preservatives can enhance the safety and efficacy of such products during their shelf life by controlling microbial growth (Roesti *et al*., 2019). Raw materials of animal origin should be critically checked before use in manufacturing batch. *S. lentus* is present in food producing animals and the staff working around them while *P. luteola* is found in damp environment and *S. warneri* on normal skin of human and animals. Products contaminated with these pathogens reflect poor handling during manufacturing process.

Conclusion: The study revealed that non-sterile pharmaceutical products are not manufactured by proper aseptic process so are not free from microbial contaminants. Poor adherence to cGMP, such as poor quality control of manufacturing area environment, improper gowning of workers and contaminated primary packaging materials. Ultimately by the time of their use, a higher microbial growth persists in such products which affects the product efficacy. Pharmaceutical industries should ensure that every liquid dosage form undergoes microbial limit test. In addition, steps should be taken to regularize the marketing of non-registered herbal liquid dosage forms.

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