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EVALUATION OF PRESERVATIVE EFFECTIVENESS OF FERULIC ACID DERIVATIVES IN ALUMINIUM HYDROXIDE GEL- USP

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ABSTRACT: The selected amide and ester derivatives of ferulic acid were subjected to preservative efficacy testing in an official antacid preparation, (Aluminium Hydroxide Gel-USP) against *Staphylococcus aureus, Bacillus subtilis, Escherichia coli, Candida albicans* and *Aspergillus niger* as representative challenging microorganisms as per USP 2004 guidelines. The selected derivatives were found to be effective against all selected strains and showed preservative efficacy comparable to that of standard and even better in case *B. subtilis* and *C. albicans*. The 8- hydroxy quinoline ester derivative showed better preservative efficacy than standard as well as other derivatives and have better potential for use in the pharmaceutical preparations.

INTRODUCTION: Deterioration of either food or pharmaceutical preparations due to growth of microorganisms is a great challenge and need of preservation becomes very important [1].

Non-sterile products such as pharmaceuticals, cosmetics, food items etc. with a high degree of water availability may be contaminated with microorganisms which may cause spoilage of the product with loss of therapeutic properties and, if they are pathogenic, serious infections can arise [2]. To inhibit the growth of contaminating microorganism, antimicrobial preservative systems have been developed and introduced into the pharmaceutical, cosmetic or food products during manufacturing process and/or throughout its use by consumers [3].

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In several cases, the microorganisms became resistant to antimicrobials and in some cases are able to degrade many commonly used preservatives especially *p*-hydroxybenzoates, e.g., parabens [4]. Preservatives to which resistance has been reported includes benzoic acid, benzalkonium chloride, chloramine, chlorhexidine, cholorophenol, dibromodicyanobutane, dimethyl oxazolidine, dimethyl dithiocarbamate, dimethoxy dimethyl hydantoin, formaldehyde, glutaraldehyde, hydrogen peroxide, iodine, mercuric salts, methylene bischlorophenol, methylparaben, propylparaben, phenylmercuric acetate, povidine-iodine, quaternary ammonium compounds and sorbic acid [5].

Also, the commonly used chemical preservatives may cause very serious side effects such as the benzalkonium chloride may cause mucosal damage and was also reported as genotoxic and cytotoxic [6, 7]. Thiomerosal used in ocular and nasal preparations was reported to be cytotoxic by Liao *et al.,* 2011 [8]. The use of parabens may cause skin cancer, genotoxicity and breast cancer as reported by the study of Dabre *et al.,* 2008 [9].

The United States and British pharmacopoeias describe official methods for evaluation of preservative system [10, 11]. Preservative efficacy test (challenge test) involves the artificial introduction of representative microorganisms including Gram positive and Gram negative bacteria, mould and yeast into the product under study, in sufficient amounts followed by the collection of kinetic information regarding the loss of their viability.

The preservative potential of natural organic acids is well established in the literature *viz.* capryllic acid [12], veratric acid [13], 2, 4 hexadienoic acid [14] and anacardic acid [15]. Literature reports reveal that the ferulic acid possesses antimicrobial, antioxidant and preservative activities [16, 17].

In view of the potential of microorganisms developing resistance to most common preservatives it became imperative to develop newer and stronger preservatives.

Further, in view of the reported toxicity potential of common synthetic preservatives, it would be quite judicious to develop the preservatives based on the natural sources such as ferulic acid.

In this context, amide and ester derivatives of ferulic acid were investigated for preservative efficacy in the present work. The preservative efficacy of most effective amide and ester derivatives of ferulic acid against gram positive *Staphylococcus aureus MTCC 2901*, *Bacillus subtilis MTCC 2063*, gram negative *Escherichia coli MTCC 1652*, fungal strains *Aspergillus niger MTCC 8189* and *Candida albicans MTCC 227* was investigated and compared them with the standard preservatives methyl and propyl paraben, in Aluminium Hydroxide Gel –USP [18].

MATERIALS AND METHODS:

Materials: Nutrient agar, nutrient broth, sabouraud dextrose agar and sabouraud dextrose broth were obtained from Himedia, Mumbai. Mannitol, methyl and propyl paraben were obtained from CDH, Mumbai.

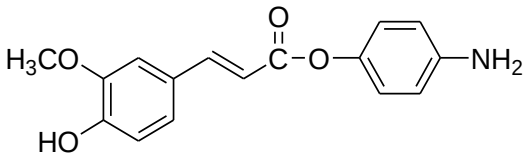
Methods: Aluminium Hydroxide Gel USP was used as the pharmaceutical product for evaluation of preservative efficacy testing.

Formula for preparation of Aluminium Hydroxide Gel USP 2004: Aluminium Hydroxide Gel, 36 g; Mannitol, 7 g; Methyl paraben, 0.2 g; Propyl paraben, 0.02 g; Saccharin, 0.05 g; Peppermint oil, 0.005 ml; Alcohol, 1 ml; Purified water q.s., 100 ml. The weighed quantity of aluminum hydroxide gel and mannitol were triturated with 50 ml of water in a mortar. Methyl paraben, propyl paraben, saccharin and peppermint oil were dissolved in alcohol and added to above mixture and triturated well. The volume was made up to 100 ml with purified water followed by its sterilization by autoclaving.

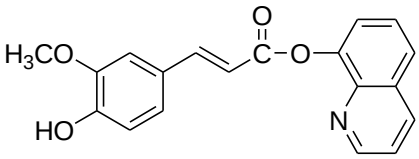
For preservative efficacy testing, the Aluminium Hydroxide Gel was prepared using the preservatives mentioned in Table 1 by replacing methyl paraben and propyl paraben from the above formula. The equimolar amount of selected preservatives (**Fig. 1**) were calculated with reference to the amount of methyl paraben (0.0013 mol) and added into aluminum hydroxide gel [19].

TABLE 1: AMOUNT OF SELECTED PRESERVATIVES ADDED IN ALUMINUM HYDROXIDE GEL – USP

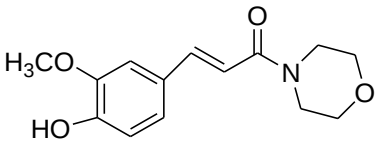
S. No.	Preservative	Amount (g)
1.	Ferulic-p-amino ester	0.370
2.	Ferulic-morpholino amide	0.341
3.	Ferulic 8-hydroxy quinoline ester	0.417
4.	Ferulic naphthyl amide	0.414



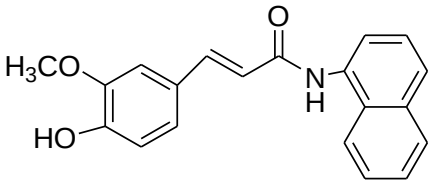
FERULIC-P-AMINO ESTER



FERULIC NAPHTHYL AMIDE



FERULIC-MORPHOLINO AMIDE



FERULIC 8-HYDROXY QUINOLINE ESTER

FIG. 1: STRUCTURES OF SELECTED FERULIC ACID DERIVATIVES

Strains: *Staphylococcus aureus MTCC 2901, Bacillus subtilis MTCC 2063, Escherichia coli MTCC 1652, Candida albicans MTCC 227* and *Aspergillus niger MTCC 8189* were used in this study were common contaminants and prescribed in USP for preservative efficacy testing in pharmaceutical preparations.

Preservative efficacy testing in Aluminium Hydroxide Gel USP 2004: The preservative efficacy test was performed essentially following the standard protocol described in USP-2004. In all cases the preservative efficacy test was done in Aluminium hydroxide gel-USP with and without the preservative system. The unpreserved product was used as a control to evaluate the viability of the inoculated cells and their ability to grow in the product.

Preparation of inoculum: The representative microorganisms were inoculated in nutrient agar I.P. (*S. aureus, B. subtilis, E. coli*) and sabouraud agar I.P. (*C. albicans, A. niger*). The seeded plates were incubated at 37^{o}C for 24 h (*S. aureus, B. subtilis* and *E. coli*), 37^{o}C for 48 h (*C. albicans*) and 25^{o}C for 7 d (*A. niger*). After the incubation period, suspensions of microorganisms were prepared in sterile saline solution (0.9% w/v NaCl) to give a microbial count of 1x 10^4 CFU/ml[13].

Test Procedure: Aluminium hydroxide gel-USP in their final container was used in the challenge test. The preparation was inoculated with the microbial cell suspension with a cell count of 1 x 10^4 CFU/ml. The inoculum never exceeded 1% of the volume of the product sample. Inoculated samples were mixed thoroughly to ensure homogeneous microorganism distribution and incubated. The CFU/ml of the product was determined at an interval of 0, 7, 14, 21 and 28 d on agar plate. The log values of number of CFU/ml (**Table 2 to Table 6**) of Aluminium Hydroxide Gel was calculated and compared as per the guidelines of USP 2004.

Criteria of acceptance for preservative system: As per USP 2004 requirement for antacid made with an aqueous base, preservative effectiveness is met if there is no increase from initial calculated count at 14 and 28 d in case of bacteria, yeast and moulds and where, no increase is defined as not more than 0.5 $\log_{10}$ higher than previous value measured (USP 2004).

RESULTS AND DISCUSSION: The results of preservative efficacy testing performed in triplicate were reported as mean values in Table 2 to Table 6. In case of *B. subtilis,* among the esters of ferulic acid, the p-amino and 8-hydroxy quinoline esters showed less than 0.5 log values of increment of CFU/ml at 14 d and 28 d and hence, both passes the preservative efficacy test. These results of 8-hydroxy quinoline were also supported by the study of Judge *et al.*, 2008 [14].

Among the amide derivatives of ferulic acid, naphthyl amide was active on 14 and 28 d but the morpholine amide fails to meet the required limit on 14 d. Also, the standard passes the preservative efficacy test on 14 d but fails on 28 d as the change was more than 0.5log CFU/ml (**Table 2**).

TABLE 2: BACTERIAL COUNT OF *B. SUBTILIS* IN ALUMINIUM HYDROXIDE GEL-USP SUPPLEMENTED WITH PRESERVATIVES

Preservative added	Log CFU/ml (Time in days)				
	0	7	14	21	28
Ferulic-p-amino ester	0.850	1.222	1.125	0.819	0.865
Ferulic-morpholino amide	1.084	1.699	1.038	0.873	0.763
Ferulic 8-hydroxy quinoline ester	0.424	0.699	0.339	0.505	0.497
Ferulic naphthyl amide	0.753	0.959	0.849	0.748	0.699
Standard	0.602	0.477	0.000	0.000	0.778
Control	0.698	0.602	1.113	0.301	0.845

In case of *S. aureus* all the esters and amide derivatives of ferulic acid and standard meets USP 2004 guidelines for preservative effectiveness testing, but the naphthyl amide derivative of ferulic acid showed more than 0.5 log values on 14 d and hence failed to meet the required limit but on 28 d the same derivative showed the slight increase in log values and hence was less potent as compared to others (**Table 3**).

TABLE 3: BACTERIAL COUNT OF *S. AUREUS* IN ALUMINIUM HYDROXIDE GEL-USP SUPPLEMENTED WITH PRESERVATIVES

Preservative added	Log CFU/ml (Time in days)				
	0	7	14	21	28
Ferulic-p-amino ester	0.377	0.681	0.572	0.623	0.788
Ferulic-morpholino amide	0.523	0.903	0.651	0.720	0.900
Ferulic 8-hydroxy quinoline ester	0.076	0.380	0.236	0.281	0.477
Ferulic naphthyl amide	0.921	1.778	0.873	0.720	0.852
Standard	0.602	0.301	0.000	0.301	0.477
Control	0.903	0.477	0.602	0.778	0.845

As per the result shown in **Table 4**, all the ester and amide derivatives of ferulic acid were found to be active against *E. coli* on 14 as well as on 28 d and met the requirement for preservative efficacy testing as per USP 2004.

In case of *C. albicans*, the amide and ester derivatives of ferulic acid showed less than 0.5 log CFU/ml from 7 to 28 d, hence they passes the preservative effectiveness test. There was decrease in log CFU/ml from 7 to 14 d in case of *p*-amino ester derivative of ferulic acid that was more than 0.5 log values and hence its efficacy was less as compared to other derivatives against *C. albicans*. Also, the log CFU/ml values of standard exceeded the prescribed USP 2004 criteria on 28 d and hence the standard was less effective preservative as compared to the synthesized esters and amide derivatives of ferulic acid against *C. albicans* (**Table 5**).

TABLE 4: BACTERIAL COUNT OF *E. COLI* IN ALUMINIUM HYDROXIDE GEL – USP SUPPLEMENTED WITH PRESERVATIVES

Preservative added	Log CFU/ml (Time in days)				
	0	7	14	21	28
Ferulic-p-amino ester	1.046	0.802	1.038	0.829	0.921
Ferulic-morpholino amide	0.509	0.103	0.426	0.528	0.456
Ferulic 8-hydroxy quinoline ester	0.699	0.200	0.535	0.829	0.602
Ferulic naphthyl amide	0.444	0.473	0.903	0.954	1.046
Standard	0.778	0.327	0.602	0.302	0.698
Control	0.845	0.602	0.778	0.954	1.041

TABLE 5: FUNGAL COUNT OF *C. ALBICANS* IN ALUMINIUM HYDROXIDE GEL - USP SUPPLEMENTED WITH PRESERVATIVES

Preservative added	Log CFU/ml (Time in days)				
	0	7	14	21	28
Ferulic-p-amino ester	1.176	1.574	0.786	1.155	0.875
Ferulic-morpholino amide	1.653	0.699	0.699	0.824	0.921
Ferulic 8-hydroxy quinoline ester	1.051	0.875	0.865	0.824	0.921
Ferulic naphthyl amide	0.653	0.796	0.579	0.699	0.921
Standard	0.301	0.698	0.602	0.778	0.000
Control	0.477	0.778	0.845	0.845	0.903

As shown in **Table 6**, the change in log CFU/ml on 14 as well as on 28 d for *p*-amino ester, morpholine amide and 8-hydroxy quinoline ester derivatives was within the limits prescribed in USP 2004 but the naphthyl amide derivative showed more than 0.5 log value from 7 to 14 d and in case of standard also the change was more than the prescribed limit and hence it was less active preservative than the other esters and amide derivatives against *A. niger*. Also, these results are in accordance with the study of Ohlan *et al.*, 2008[13].

TABLE 6: FUNGAL COUNT OF *A. NIGER* IN ALUMINIUM HYDROXIDE GEL-USP SUPPLEMENTED WITH PRESERVATIVES

Preservative added	Log CFU/ml (Time in days)				
	0	7	14	21	28
Ferulic-p-amino ester	0.596	1.176	0.996	0.675	0.564
Ferulic-morpholino amide	0.699	1.000	0.899	0.913	1.041
Ferulic 8-hydroxy quinoline ester	0.875	0.778	1.034	1.000	0.895
Ferulic naphthyl amide	1.875	1.000	1.598	1.109	1.439
Standard	0.301	0.301	0.698	0.000	0.477
Control	0.698	1.079	0.954	1.000	1.079

CONCLUSION: The study has shown the preservative potential of *p*-amino ester and 8-hydroxy quinoline ester, naphthyl amide, morpholine amide of ferulic acid in the pharmaceutical preparation. The selected amide and ester derivatives of ferulic acid were found effective against all selected strains and showed preservative efficacy comparable to that of standard and even better in case of *B. subtilis* and *C. albicans*. The 8- hydroxy quinoline ester derivative showed better preservative efficacy than standard as well as other derivatives and it can be a better alternative to the existing preservatives for use in the pharmaceutical preparations.

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