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In vitro and *in vivo* potentiation of ampotericin-B by flavonoid against different fungal strains

Authors:
Zahid Mahmood[1]
Vijeshwar Verma[2]
Inshad Ali khan[3] and
Mohmmad Afzal Zargar[1]

Institution:
1. Department of Biochemistry, University of Kashmir, Srinagar, 190006, India.

2. Shri Mata Vaishno Devi University, 182320, Katra.

3. Microbiology Divisions, Indian Institute of Integrative Medicine, 180001, Jammu.

Corresponding author:
Mohmmad Afzal Zargar

ABSTRACT:

Synergistic effects of 18 flavonoids (11 glycosides and flavones, 01 flavones diglycoside, 04 chalcones and 02 aglycones) in combination with different anti-fungal agents against fungal strains were investigated. The agar diffusion assay of these flavonoids with different anti-fungal agents was tested. The Minimum Inhibitory Concentration (MIC) values of each of the flavonoids with different anti-fungal agents were determined by using checkerboard broth micro dilution assay. Flavones diglycoside (3, 5-dihydroxy flavones 7-O-b-D-glucuronide-4-O-b-D-glucopyranside) potentiated the *in vitro* and *in vivo* activity against fungal strains. The flavones diglycoside reduced MIC of amphotericin-B to one half against different fungal strains, *Candida albicans, Candida krusei, Candida parapsilosis, Candida tropicalis* and *Cryptococcus neoformans* 1202. Although moderate change between *in vitro* and *in vivo* studies have been found, the elucidation of the mechanisms involved in flavonoid action will have many health benefits to man. In conclusion, these findings suggested that flavonoid combination regimens may be considered as an useful candidate for the treatment of fungal infection.

Keywords:
Flavonoids, minimium inhibitory concentration, kill kinetics, amphotericin-B

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INTRODUCTION

Flavonoids belong to the large group of plant's polyphenols. They have prominent role in the pigmentation and protection of plants against different external agents. In recent years, there is a raising interest in flavonoids mostly because of their antioxidant, anti-inflammatory, anti-allergic, antimicrobial and anticancer activity. Flavonoids are the building block of polyphenolic compounds that can be found in various foods. They commonly have a generic structure consisting of two aromatic rings (A and B rings) linked by three carbons that are usually in an oxygenated heterocyclic ring (C ring). So far, over 4000 structurally unique flavonoids have been isolated from plant sources (Harborn and Walbuch, 2000). During 1990's, flavonoids were shown to possess several biological effects, related to human health (Harborn and Williams, 2000) making it natural to search for even more effective compounds among the sources of those phenolic compounds in the plant kingdom. There is also a possibility that the new compounds could possess a noted but stronger activity in comparison to known substances. The structural diversity of the natural compounds are greater than that by the synthetic ones (Harvey, 1999). The new flavonoid structure can be used as pharmacologically unspecific leads for molecular designing of drugs (Nahrsted, 1997). The antimicrobial and resistance modifying potentials of naturally occurring flavonoids and polyphenolic compounds have been reported in other studies by Cushnie and Lamb (2005) and Sato *et al.* (2004). The synergistic effect from the association of antibiotic and plant extracts against resistant bacteria leads to new choices for the treatment of infectious diseases. This effect enables the use of respective antibiotic when it is no longer effective by itself during therapeutic treatment (Nascimento *et al.,* 2000). The application of synergistic principle is evident in commercial preparations for the treatment of various infections (eg. the antibiotic, Augmentin). Traditional healers often use combinations of plants to treat or cure diseases (Kamatou *et al.,* 2006). Medicinal plants have been used in many forms over the years to treat, manage or control man's ailments (Prescott *et al.,* 2002). Therefore, any effort to further explore the medicinal or natural products from man's botanical flora towards improving health care delivery deserves attention . The presence of efflux pumps and Multi Drug Resistance (MDR) proteins in antibiotic resistant organisms contribute significantly to the intrinsic and acquired resistance in these pathogens (Oluwatuyi *et al.,* 2004). The discovery and development of new compounds that either block or circumvent resistance mechanisms could improve the containment, treatment, and eradication of these strains (Oluwatuyi *et al.,* 2004; Sibanda and Okoh, 2008). Combination therapy can be used to expand the antimicrobial spectrum, to prevent the emergence of resistant mutants, to minimize toxicity, and to obtain synergistic antimicrobial activity (Pankey *et al.,* 2005). One way to overcome antibiotic-resistant bacteria is through the use of new antimicrobial compounds and / or combination therapy. This study was carried out to seek an approach considered as an alternative treatment in terms of combination therapy between flavonoids and an available antibiotic against different pathogens.

Table 1. Disk diffusion assays of flavones diglycoside and standard drug AMB

Fungal cultures	Zone of inhibition (mm) (Mean)	
	AMB (1µg/ml)	Flavones diglycoside
C. albicans V-01-191	21	36
C. krusei	23	28
C. parapsilosis	27	32
C. tropicalis	28	33
C. neoformanis 1202	25	30

MATERIALS AND METHODS

A structurally diverse library of 18 flavoniods was obtained from Natural Product Chemistry Lab, Indian Institute of Integrative Medicine (IIIM) formerly known as Regional Research Laboratory (CSIR) Jammu. Amphotericin-B was purchased from (sigma Aldrich CO.St.Louis, MO, USA). The media component Sabourad

Table 2. **Combination studies of flavones diglycoside with AMB**

	MIC(µg/ml)							
Organisms	**AMB**	**AMB+0.2**	**AMB+0.4**	**AMB+0.8**	**AMB+1.56**	**AMB+3.12**	**AMB+6.25**	**AMB+12.5**
C. albicans V-01-191	0.5	0.5	0.25	0.25	0.25	0.25	0.25	0.25
C. krusei	0.25	0.25	0.25	0.12	0.12	0.12	0.12	0.12
C. parapsilosis	0.5	0.5	0.25	0.25	0.25	0.25	0.25	0.25
C. tropicalis	0.5	0.5	0.5	0.25	0.25	0.25	0.25	0.25
Cryptococcus neoformanis 1202	0.25	0.25	0.12	0.12	0.12	0.12	0.12	0.12

Dextrose agar (Becton-Dicknson) and RPMI (Sigma, Steinheim, Germany) were purchased for the culture of fungi strains. The fungal strains used in this study *viz Candida albicans* V-01-191, *Candida krusei* ATCC, Candida parapsilosis ATCC, *Candida tropicalis* ATCC and *Cryptococcus neoformans* 1202 were obtained from the American Type Culture Collection (*Manassas. Va*.). To evaluate the general antifungal activity of the flavoniods, agar diffusion assay was used. Stock solution of 1mg/ml of flavonoid and antifungal drug was prepared in DMSO/distilled water. Sabourad Dextrose Agar (SDA) was prepared for culturing fungal strains. 500 µl from 0.5 Mcfarland of the suspension was standardised by adjusting the optical density to 0.1 at 600 nm wavelength (Shimadzu UV-vis spectrophotometer). It was poured in the agar flask and mixed and poured into PD150 mm sterile plastic plates. The plates were set down and 6mm wells were punched. 50µl of respective dilution were pipetted into the wells. Plates were incubated at 37°C for 24 h. Microbial growth inhibition was determined by measuring the zones of inhibition using a transparent ruler. Evaluations of the susceptibility of fungal cultures were made by the micro broth dilution method as per NCCLS document M27-A (NCCLS, 2000). The fungi used as inocula were grown overnight on Sabouraud dextrose agar at 37°C for 24 h. Tests were performed in RPMI 1640 (Gibco-BRL) buffered to pH 7.0 with 0.165 M Morph Oline Propane Sulphonic acid (MOPS; Sigma). Inoculum effects were determined as per NCCLS (2000), except that strains were suspended to a turbidity equivalent to that of a 0.5 McFarland standard in 0.9% (w/ v) NaCl and were further diluted in 0.9% NaCl to achieve the desired inoculum levels. Inoculum densities were verified by determining the number of viable colonies per millilitre on Sabouraud dextrose agar after serial dilutions in 0.9% NaCl. The stock solution of the flavoniods and antifungal drug were prepared in DMSO /distilled water and was serially diluted in the microtitre plates by two fold dilutions of antifungal drug in combination with two fold dilutions of flavoniods in U-bottom 96-well microtitre plates. The final concentrations of antifungal agents ranged from 0.12µg/ml to 64µg/ml and for flavonoids from 0.8µg/ml to 50µg/ml. The cultures were then diluted to 1:50 in normal saline and 1:20 in RPMI media to get final inoculums (1-2 × 10^3 cfu/ml). Each well of the microtitre plate was then inoculated with 100µl of diluted inoculum and incubated at 37°C for 48 h. The MIC was considered to be the lowest concentration of the compound that inhibited the visible growth of fungi.

Time - kill experiment

Determination of the rate of kill of the flavones diglycoside was done following the procedure described by Aiyegoro *et al.* (2008) with slight modifications. Amphotericin-B was tested at 0.25 and 0.5mg/ml respectively. Flavones diglycoside at a concentration of 50mg/ml was combined with amphotericin-B at a concentration of 0.25 and 0.5mg/ml. Amphotericin-B was also tested alone at 50mg/ml. Time-kill studies were performed at an inoculum of 2 x 10^6 colony forming unit in 20 ml volume of the medium. The flasks were incubated at 37°C on an orbital shaker at 120 rpm. One flask of inoculated Sabouraud dextrose broth with no drug served as a control. Colony counts were performed on the control suspension at time zero and on the control as well

Table 3. *In-vivo* efficacy of flavones diglycoside in combination with AMB in mice Infection model

Name of the organism : *Candida albicans* V-01-191
CFU/mouse : 1.2 ×10^7
No of mice : 6
Dosing schedule : IP× OD × Day0, Day2
Observation period : 14 Days

Treatment Groups	Mean Survival Days	ED_{50} mg/kg (3 Replica)
Negative control	14	
Infection control	1	
Flavone diglycoside control	1	
AMB - 0.12mg/kg	1.5	0.32
AMB – 0.25mg/kg	7.5	
AMB – 0.5mg/kg	12	
AMB - 0.12mg/kg +Flavone diglycoside -10mg/kg	2.33	0.20
AMB – 0.25mg/kg +Flavone diglycoside -10mg/kg	11.83	
AMB – 0.5mg/kg + Flavone diglycoside – 10mg/kg	12.66	
AMB - 0.12mg/kg+ Flavone diglycoside- 40mg/kg	9.16	0.15
AMB – 0.25mg/kg +Flavone diglycoside -40mg/kg	13.66	
AMB – 0.5mg/kg + Flavone diglycoside- 40mg/kg	12.66	

as each drug containing suspension at 0. 2, 5, 10 and 24 h. Suitable dilutions were made in 0.9% sterile normal saline; and 20ml was plated in triplicate on Sabouraud dextrose agar plate. The plates were incubated at 37°C for 18 to 24 h, and colony counts were performed. Kill curves were constructed by plotting $\log_{10}$ colony forming unit per milliliter against time over 24 h . Fungicidal activity was defined as a > 3-$\log_{10}$ reduction in colony count compared to the time zero count.

***In-vivo* study**

Swiss albino mice were used to evaluate the antifungal potentiation of flavonoids under study. The fungal culture used in the *in vivo* study is *Candida albicans* V-01-191 grown overnight on Sabouraud Dextrose Agar (SDA). Milky suspension was prepared in the sterile normal saline and then diluted as 1:50 and its Optical Density (OD) measured at 550nm = 0.4100 was taken. Its original suspension was diluted to 1:5, and 200µl containing 1.2× 10^7cfu/ml was injected to each mouse via lateral tail vein. The treatment was through intraperitoneal route within one hour after the infection and after two days of infection. The mice were observed daily for 14 days and the number of survival was counted. ED_{50} was calculated by Reed and Muench method (Reed and Muench, 1938) at the day 14.

Treatment group:

Group I	Group II	Group III
AMB group	AMB + flavonoid	AMB + flavonoid
0.12mg/kg	0.12mg/kg + 10mg/kg	0.12mg/kg + 40mg/kg
0.25mg/kg	0.25mg/kg + 10mg/kg	0.25mg/kg + 40mg/kg
0.50mg/kg	0.50mg/kg + 10mg/kg	0.50mg/kg + 40mg/kg

RESULTS AND DISCUSSION

Antifungal activity of flavones diglycoside was determined by agar diffusion assay and amphotericin-B was taken as a standard drug. The zone of inhibition was seen with *Candida albicans* V-01-191, *Candida krusei* ATCC, *Candida parapsilosis* ATCC, *Candida tropicalis* ATCC and *Cryptococcus neoformans* 1202 (Table-1). In micro dilution assay, the flavonoid combined with antifungal agents potentiated the antifungal activity of amphotericin-B against the strains Candida *albicans* V-01 -191, *Candida krusei* ATCC, *Candida parapsilosis* ATCC, *Candida tropicalis* ATCC and *Cryptococcus neoformans* 1202 reduced the MICs of amphotericin-B to one half in the combination studies (Table 2). However, this combination was not found to be effective against filamentous pathogen like *Aspergillus fumigatus.* Time kill studies were performed on *C.albicans* V-01-191. The growth curve of flavones diglycoside alone at 50mg/ml was almost overlapping with the growth control curve

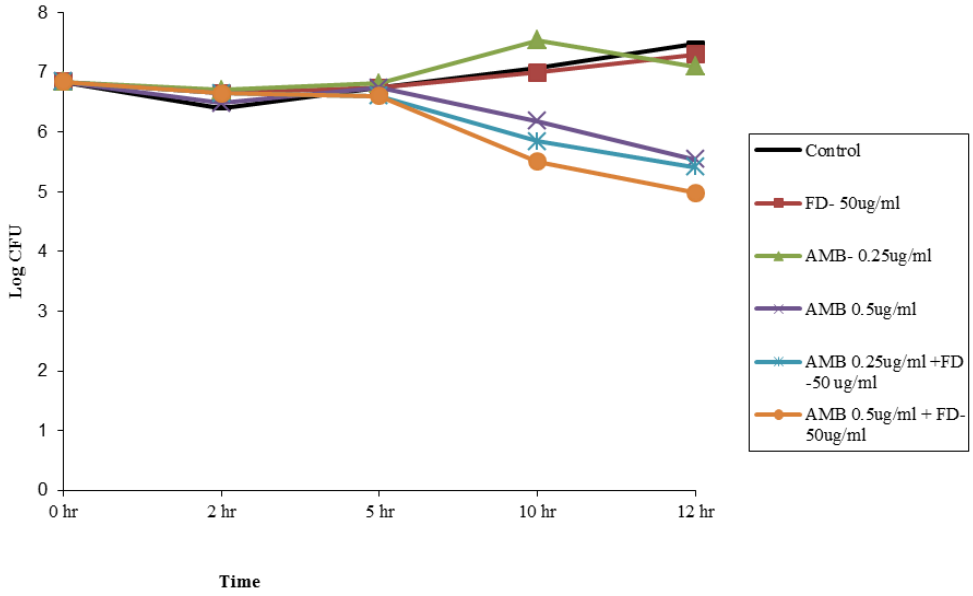

Figure 1. Kill Kinetic of *C. albicans* V-01-191

indicating that flavones diglycoside alone was not associated with any antifungal activity. Amphotericin-B brought about 99.9% kill or three log reductions at 0. 5mg/ml (Figure 1), whereas in combination with flavones diglycoside at 50mg/ml. Amphotericin could bring about the same level of killing at half the concentration i.e. 0.25mg/ml. The level of killing with amphotericin at 0.5mg/ml and the same level of killing was achieved with amphotericin at 0.25mg/ml in combination with flavones diglycoside. The activity of the flavones diglycoside in the present study was not only inhibitory to the fungi but also fungicidal. Such activities have been previously reported for other plants and the extent of the fungicidal activity has been evaluated by the time-kill experiments (Rukayadi *et al.,* 2006). Studies by Okemo *et al.* (2001) indicated that the crude extracts of the neem plant *Azadirachta indica* killed a whole population of *C.albicans* at a concentration of 8mg/ml in 24 h while Patel and Coogan, (2008) found that *Dodonaea viscose* extracts killed all the *C. albicans* strains within 30 s. In the present study, flavones diglycoside was able to completely kill *C. albicans* V-01-191 cell at a concentration of 0.25mg/ml. This indicates the possibility of compounds from this flavoniods to kill fungal organisms with special reference to *Candida sp*. at lower concentrations than the crude extract. This activity could be due to the compounds such as muzigadial and warburganal previously isolated from this plant in other studies (Rabe and Van Staden, 2000).

Flavones diglycoside (3` 5-hydxoxy flavones 7 – O –b -D-glucuronide - 4`- O – D -glucopyranside) was taken for *in-vivo* systemic model of infection against *candida albicans* in mice in combination with amphotericin-B. ED_{50} of amphotericin-B group was 0.32mg/kg, whereas ED_{50} of amphotericin-B + flavones diglycoside at 10mg/kg and 40mg/kg was 0.20mg/kg and 0.15mg/kg. It was found that amphotericin-B in combination with 40mg/kg of flavones diglycoside was more efficacious and yielded an ED_{50} of 0.15mg/kg, which was half of the ED50 value of 0.32mg/kg with amphotericin-B group alone (Table 3). However, these combination identities did not proved to be efficacious when tested in other fungal infection model such as systemic *Aspergillus* and *Cryptococcus* infection. However, in combination studies it has been found that the bacteriostatic as well as bactericidal agents at low

concentration prevent the emergence of drug resistance. Continued and further exploration of plant antimicrobials needs to occur because plant based antimicrobials have enormous therapeutic potentials. They are found effective in the treatment of infectious diseases despite its various side effects that are often associated with the synthetic drugs. Various reports have documented the enhanced antimicrobial activities (that is, synergistic potentials) of standard antibiotics in combination with plant flavonoids even when the organisms are no more susceptible to the drug. Synergistic interactions are of vital importance in phytomedicine, to explain the efficacy of apparently low doses of active constituents in a herbal product. This concept, that a whole or partially purified flavonoid of a plant offers advantages over a single isolated ingredient that underpins the philosophy of herbal medicine. Both literature reports and ethnobotanical records indicate a general consensus on the use of antimicrobials from active medicinal plants to provide cheaper drugs that may complement existing supplies from orthodox medicine in the Primary Health programme and/or provide novel or lead compound that may be employed in controlling infections in our communities (Betoni *et al.,* 2006). The potential of flavoniods as anti-microbial agents separately or in combination with the known agents, have not been explored so far. In the present study, these molecules were screened for their antifungal activities individually as well as in combination with the available antifungal agents to study the potentiation of the known antifungal agents, and achieve the advantages in combination. Tremendous therapeutical and commercial potential exists in the antifungal mycotic agents of flavonoids. But the need of the hour is to tap valuable natural resources containing these valuable flavonoids. In new antifungal drug targeting strategies, flavonoids should be given prime importance because majority of the promising antifungals may generate new drug candidates.

Time kill curves of Amphotericin alone and in combination with Flavones diglycoside. Amphotericin was tested alone at 0.25 and 0.5µg/ml in combination with Flavones diglycoside. Amphotericin was tested at 0.25, 0.5mg/ml, whereas Flavones diglycoside was combined at the concentration of 50mg/ml. Flavones diglycoside alone at 50mg/ml was also tested in order to rule out its inhibitory effect on the growth of the fungi.

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