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Artemisia annua and artemisinins are ineffective against human *Babesia microti* and six *Candida* sp

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Abstract

Background: *Artemisia annua* L.is a well-established medicinal herb used for millennia to treat parasites and fever-related ailments caused by various microbes. Although effective against many infectious agents, the plant is not a miracle cure and there are infections where it has proved ineffective or limited. It is important to report those failures.

Methods: Here artemisinin, artesunate and dried leaf slurries of *A. annua* were used daily for 6 days *in vivo* against *Babesia microti* in mice 2 days post infection at 100 μg artemisinin/kg body weight. Parasitemia was measure before and 15 days days post treatment. Artemisinin and extracts of *A. annua* also were tested *in vitro* against six *Candida* sp. at artemisinin concentrations up to 180 μM and growth measured after cultures were fed drugs once at different stages of growth and also after repeated dosing.

Results: *A. annua*, artesunate, and artemisinin were ineffective in reducing or eliminating parasitemia in *B. microti*-infected mice treated at 100 μg artemisinin/kg body weight. Although

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the growth of exponential cultures of many of the tested *Candida* sp. was inhibited, the response was not sustained and both artemisinin and *Artemisia* were essentially ineffective at concentrations of artemisinin at up to 180 μM of artemisinin.

Conclusions: Together these results show that artemisinin, its derivatives, and *A. annua* are ineffective against *B. microti* and at least six species of *Candida*.

Keywords

Artesunate; artemether; dihydroartemisinin; babesiosis; dried leaf *Artemisia annua*; dried leaf *Artemisia annua* extract

Introduction

Artemisia annua L., a medicinal plant with more than a 2 millennia history in the Chinese Materia Medica, is best known for producing the antimalarial sesquiterpene lactone, artemisinin (AN), and delivered as artemisinin combination therapy (ACT) to preclude emergence of AN drug resistance. The 2015 Nobel Prize for Medicine was awarded to Dr. Tu for her isolation and validation discovery of the antimalarial molecule in *A. annua* (1). Beside their antimalarial activity (2–4), both the plant and AN and its derivatives also showed efficacy against a number of viruses (5) including SARS-CoV-1 (6) and SARS-CoV-2 (7–9), human cancers (5,10), schistosomiasis (11), leishmaniasis (12,13), New- and Old-World trypanosomiases (14), babesiosis (15), tuberculosis (16), and many livestock diseases (17). Both AN and *A. annua* also have immunomodulatory effects, e.g. on TNF-α and IL-6, as recently shown in rats (18). Because of its low yield in *A. annua* and isolation and purification costs of AN, the drug is not affordable or inaccessible for many people living in developing countries where malaria and other diseases are problematic. In many cases, however, *Artemisia* extracts and *per os* delivered powdered leaves (DLA) are equal to or more effective than artemisinin itself in their antimicrobial effect. For example, dichloromethane (DCM) extracts of *A. annua* were significantly more potent against virulent *Mycobacterium tuberculosis* than an equimolar amount of AN (16), and hot water extracts of *A. annua* and *A. afra* (the latter produces no artemisinin) significantly reduced malaria trophozoites *in vitro* (2). While the growing list of susceptible microbes is impressive, it is crucial to identify those microbes that seem impervious to AN and/or *A. annua*.

Babesiosis is an emerging tick-borne disease threatening elderly, spleenectomised and immunocompromised people. The disease, caused by haemotropic apicomplexan protozoa of the genus *Babesia*, is transmitted mostly by *Ixodes* ticks but also prenatally and by blood transfusion (19–24). After trypanosomes, *Babesia* sp. are the most common parasites in mammalian blood (22). Babesiosis is a malaria-like disease invading erythrocytes causing symptoms like those of malaria including fever, flu-like symptoms, chills, headache, anemia, hemoglobinuria, and myalgia. In regions where malaria is endemic, co-infection with *Babesia* was observed and possibly misdiagnosed as malaria (25). Like malaria, the life cycle of *Babesia* requires an invertebrate host for sexual reproduction and transmission to the vertebrate host. After gametogenesis in a tick's midgut the motile embryo enters rounds of replication in different tissue, among these tissues are the ovaries and salivary glands the main sources for trans-ovarian-within-vector transmission and transmission into the next

vertebrate host, respectively. Unlike malaria and *Theileria*, babesia sporozoites directly invade erythrocytes without the need for replication in hepatocytes or lymphocytes. Currently the preferred anti-babesia treatment in immunocompromised patients is the combination of atovaquone and azithromycin, however relapse occurs after prolonged treatment, suggesting *B. microti* are resilient to such a combination therapy and survive the treatment especially in immunocompromised patients (26). Mice are a good model to study efficacy of *A. annua in vivo* against *B. microti* (23).

Of the 1.5 million annual global deaths due to fungal infections, 30–40% are attributed to invasive *Candida albicans*, a normally opportunistic fungus existing in a commensal relationship with the human body (27). Infections by other species such as *C. glabrata, C. tropicalis*, and *C. lusitaniae* have also grown in number (28); *C. aureus* is particularly notable because it has become more widespread and drug resistant (29).

In 2005 Galal *et al.* (30) reported antifungal activity of 29 AN derivatives against ATCC 90028 strain *C. albicans*; six had antifungal activity within 50 μg/mL. The effective derivatives included artemisinin (AN), dihydroartemisinin (DHA), anhydroDHA, β-arteether, α-arteether, and a deoxyartemisinin derivative, with IC_{50} values ranging from 3–30 μg/mL. Of these, only AN, anhydroDHA, and β-arteether, completely inhibited growth of the fungus at 50 μg/mL; the drugs appeared to be fungistatic rather than fungicidal. De Cremer *et al.* (31) studied the efficacy of AN and AN derivatives including artesunate (AS), artemether (AM), and DHA against SC5314 wild type strain *C. albicans* biofilm formation ± miconazole, a drug currently used to treat *C. albicans* infections. AS in particular had a synergistic effect, reducing the concentration of miconazaole required to decrease metabolic activity of the fungal biofilm by half. AS concentrations as low as 20 μM produced a slight accumulation of ROS that further increased with miconazole treatment of the biofilm cells. The synergistic activity between the artemisinic derivatives and miconazole suggested that the endoperoxide bridge characteristic of artemisinin drugs plays a key role in reducing biofilm metabolic activity.

Together these studies suggested a further investigation of *A. annua* for treating babebiosis and candidiasis was warranted. Here we report on the response of six *Candida* species and human *B. microti* to AN, AN derivatives, and to *A. annua* extracts and orally gavaged leaves (DLA), respectively. We present the following article in accordance with the ARRIVE reporting checklist (available at https://dx.doi.org/10.21037/lcm-21-2).

Methods

Biological test species cultivation

Artemisia annua L. (SAM cultivar; voucher MASS 00317314) containing 1.48%±0.06% (w/w) artemisinin, 0.37% flavonoids, as determined by GC-MS was used in this study. Plants were grown, harvested, dried, and leaves sieved and pulverized as previously described (4). *A. annua* extracts were prepared from dried leaves of two species: DLAeS (voucher MASS 00317314), a high artemisinin-producing cultivar (32) and glandless (DLAeG; voucher 171772 and 170353), an artemisinin null mutant (33). Six *Candida* species were tested. *C. albicans* (SC5314), *C. tropicalis* (MYA-3404), *C. glabrata* (34), *C.*

lusitaniae (ATCC 42720), *C. dubliniensis* (34), and *C. parapsilosis* (CDC 317) were maintained on YPD 2% agar plates, and subcultured on fresh YPD plates every two weeks. For some experiments, *Candida* sp. were grown in liquid SC media in a New Brunswick Scientific TC-7 roller drum incubator at 30 ℃, 1% carbon dioxide and 28 rpm for 12–16 hs and growth measured at 600 nm. The 90 min doubling time of *Candida* in SC media with an OD of 1 at 600 nm was validated at ~5×10^7 cells (35,36) and was used to quantify growth. Cryopreserved *B. microti* (GI) strain used in the *in vivo* experiments was kindly provided by Samuel Telford, Tufts University, Medford, MA, and was isolated in 1981 from a patient who acquired the infection on Nantucket Island (R), Massachusetts, USA (37).

Babesia in vivo challenge

B.microti parasites were intraperitoneally injected into two inbred male DBA/2 mice for activation after cryopreservation in liquid nitrogen. Infected red blood cells were collected from the first activation passage 14 days after inoculation and 10^7 infected red blood cells were used to infect two mice for another round of activation. The activated parasites were harvested and then used in the *in vivo* challenge. Inbred 12 week old male DBA/2 mice were intraperitoneally injected with 10^7 infected red blood cells and randomly divided into four groups AN, artesunate (AS), dried leaf *A. annua* (DLA), and placebo with six mice in each group. Two days after parasite inoculation, mice were treated daily for six days via oral gastric gavage of (100 mg/kg) of either AN or AS and 167 mg DLA in water, forming a slurry material corresponding to 100 mg/kg AN. AN and AS were prepared in a water slurry using 167 mg mouse chow per mouse. Mice in the placebo group were gavaged as a water slurry of mouse chow. Details about mouse housing, feeding, drug preparation, parasitemia count, and euthanasia were reported previously (4). Parasitemia was measured daily on Giemsa-stained thin blood smears and mice were euthanized 10 days after last treatment on day 17 post inoculation. Average parasitemia decline ≥20% of the placebo triggered analysis of statistical significance.

A. annua and drug preparation and analysis

To prepare an extract of *A. annua*, 35 g of dried plant leaves (combined harvested lots 2012–2015 for DLAeS and 2013–2014 for DLAeG) were aliquoted into 1 g samples in separate 50 mL test tubes to which 20 mL of methylene chloride were added prior to water bath sonication for 30 min at room temperature. Extract was separated from residual plant solids, pooled and evaporated under N_2 at 30 ℃. Extraction was repeated twice, pooled, filtered through glass wool in a Pasteur pipette, evaporated under N_2 and stored at −4 ℃ until use. AN was analyzed using GC-MS and quantified using authentic AN according to the method detailed in Martini *et al.* (16). Artemisinin (AN), artemether (AM), artesunate (AS), and dihydroartemisinin (DHA) were ordered from Cayman Chemical and solubilized in 100% filter sterilized DMSO to produce master stock solutions of 70 mM for *Candida* experiments.

Candida halo assay screen

Drugs and extracts were initially screened for activity using the halo inhibition assay on agar plates. The six *Candida* sp. were grown in SC media for ~16 h, diluted to an OD of 10^{-3}, and 500 μL were spread on SC media 4% agar plates; each plate contained approximately

1×10^4 cells. After 1 h drying, a 1 cm diam, #1 Whatman filter paper disk was placed onto each quadrant of each plate and infused with one of the following: 0.7 μM of AN, AS, DHA, or AM; 84.7 μg of dry weight DLAeS, or a dry mass of DLAeG equal to the mass of DLA-SAM that yielded 0.3 μM AN; 10 μL of 100% DMSO was added to the negative control with no drug. The AN equivalent of 0.3 μM DLAeS and DLAeG was also tested. The quantities of drug and extract used were determined such that the maximum quantity allowed by their solubility limits was fully infused into the filter paper disks. Plates incubated at 30 ℃ in 1% carbon dioxide were analyzed after 16–20 h incubation when there were visible zones of inhibition around the filter disks.

Drug and A. annua effects on Candida growth

Candida cultures were prepared with 0.01–1,000 μM of DLAeS and each AN-derivative drug that inhibited the four *Candida* strains in the halo assay screen: *C. albicans, C tropicalis, C. glabrata,* and *C. lusitaniae.* Drugs and extracts were delivered in 3% DMSO, the highest DMSO concentration that did not impair growth and served as a negative control. Average % growth was normalized to the DMSO control for each tested drug concentration. Mid and stationary log phases were at 16 and 28 h post inoculation. Drug and DLAe impact were then measured for each species grown for 28 h at the IC_{50}. DLAeG contains no AN, so growth was measured against the biomass equivalent of DLAeG corresponding to the biomass of DLAeS at its IC_{50}. The IC_{50} for each tested AN-derivative drug and DLAeS was calculated using GraphPad Prism 8.

The effect of single *vs.* multiple doses of AN and DLAeS on *Candida* growth was compared. *C. albicans* cultures were prepared in duplicate in liquid SC media: a 3% DMSO negative control; and AN and DLAeS at their respective IC_{50}, 180 and 52 μM. Single dose cultures containing 180 μM DLAeS were also prepared to directly compare the antifungal effect of AN and DLAeS at the IC_{50} of AN. Multiple dose cultures were treated with a second and third dose of drug/DLAe at six and 22 hs by pelleting at 4,000 ×g, aspirating media, and resuspending in 3 mL of fresh SC media with 3% DMSO, 180 μM AN, or 52 μM DLAeS. Colony-forming units (CFUs) were measured for 20 μL aliquots from both single and multiple dose cultures. To compare fungal growth between single and multiple dose treatments, the average normalized % growth was plotted versus time in GraphPad Prism 8.

Single vs. multiple doses of AN or DLAe on Candida survival

The 20 μL aliquots collected at 16 and 28 h growth from single and multiple dose *C. albicans* cultures were each mixed with 13.3 μL of 50% 0.22 μm sterile filtered glycerol and incubated at −20 ℃ for 24 h and then −80 ℃ for 48 h. Aliquots were thawed for 1 min at 23 ℃, peleted at 4,000 ×g, and the media aspirated. Pellets were resuspended in SC media to make a 10^{-1} dilution of each fungal culture and then serially diluted into separate microfuge tubes. For technical replicates, a 2% agar YPD plate was sectioned into six parts and plated with three, spaced, 5 μL drops of each serial dilution on individual sections. Plates were incubated for 20 h at 30 ℃. Afterwards, fungal colonies were counted on the lowest fungal serial dilution section containing non-confluent colonies per treatment to give CFU/mL.

In vivo experiments were performed according to Protocol# 2011–0072 approved by the Institutional Animal Care and Use Committee (IACUC) of the University of Massachusetts Amherst, MA, USA in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the US National Institutes of Health. All efforts were made to minimize suffering of animals during experimental procedures.

Statistical analyses

Babesia experiments had six mice per treatment and averages were compared. Each *Candida* experiment had ≥3 biological replicates each having ≥2 technical replicates. Tukey's Multiple Comparisons test was used to determine statistical differences between negative controls and drug-treated fungal cultures. A two-tailed t-test assuming equal variance was used to test for statistical difference between the DLAeS- and DLAeG-exposed fungal samples and between CFUs/mole AN for AN- and DLAeS-exposed fungal cultures. Results were plotted using GraphPad Prism 8.

Results

Neither artemisinin nor A. annua inhibited B. microti

We designed our experiment to test whether DLA will have an anti-babesial activity comparable to AN and AS. Parasites appeared in the blood of infected mice 48 h after inoculation then increased gradually entering the log phase during treatment from day 2 to 7 post inoculation, reaching the peak on day 11 post inoculation and four days after last treatment. Parasitemia suddenly dropped 12–14 d post inoculation then entered a second log phage on day 15 post inoculation (Figure 1). Mice were euthanized 10 days after last treatment when parasitemia was about 15%. None of the treated groups showed significant reduction in parasitemia compared to placebo controls (Figure 1). No adverse responses were observed in any groups (see Discussion).

Candida screening and IC50 assays

Clear, distinct, and consistent halos were observed for the following species after 16 and 20 hs of growth at 30 ℃: *C. albicans, C. dubliniensis, C. lusitaniae, C. tropicalis,* and *C. glabrata*. No halos were observed for *C. parapsilosis*; data are summarized in Table 1. Tukey's Multiple Comparisons test showed no significant difference among the four species, *C. albicans, C. tropicalis, C. glabrata*, and *C. lusitaniae,* so all further tests used these four *Candida* species and were measured at the same time points at 8, 16, 20 and 28 h, respectively, for beginning log, mid-log, beginning stationary, and full stationary phases (Figure 2).

C. albicans, C. glabrata, C. tropicalis, and *C. lusitaniae* were all tested against AN, AN derivative drugs and DLAeS to determine their IC_{50}s at mid-log, 16 h (Figure 3). When normalized to the 3% DMSO control for each species, *C. albicans,* DLAeS had the lowest IC_{50} (52.0 μM) compared to AN and AM at 16 h. The IC_{50} of AN was 180.3 μM and more than twice that of DLAeS. AM had the highest IC_{50} at 219.8 μM. However, none of these IC_{50} values for *C. albicans* were significantly different from each other at $P \leq 0.05$. For *C. glabrata,* the IC_{50} of AS was significantly greater at 529.4 μM relative to the other treatments

with IC_{50} values for AM, AN and DLAeS at 74.5, 101.0 and 102.7 μM, respectively. For *C. tropicalis,* AN had a lower IC_{50} (9.37 μM) compared to DLAeS (12.08 μM), though this difference was insignificant. IC_{50} values were not significantly different for all of the tested AN drugs and DLAeS on *C. lusitaniae*.

IC_{50}s also were measured at 28 h, stationary phase, for the same four *Candida* species (Figure 4). The IC_{50} values determined at the 28 h stationary phase for AN and AS against all *Candida* species tested, for AM against *C. albicans, C. glabrata,* and *C. lusitaniae*, and DLAeS against C. albicans, *C. tropicalis*, and *C. lusitaniae* all exceeded 66 μM. The IC_{50} of AM against *C. glabrata* was 581.7 μM) and of DLAeS against *C. tropicalis* was 219.2 μM. Additionally, only the IC_{50} values for AN and DLAeS against *C. tropicalis* were significantly different from each other. All IC_{50} values are summarized in Table 2.

Of the four species tested, the AN derivative drugs and DLA extracts had the strongest antifungal effect against *C. tropicalis*, where AN had an IC_{50} of 8.4 μM and DLAeS had an AN IC_{50} of 11.1 μM (equivalent to 9.4 μg leaf dry weight) at 16 hs. Neither DLAeS nor the other artemisinic drugs were effective against the other three species at an $IC_{50} \leq 50$ μM (Table 2). By 28 h none of the four species were inhibited with an $IC_{50} \leq 50$ μM (Table 2).

Using Tukey's Multiple Comparisons test, the log phase midpoint was significantly different between the 3% DMSO- and 180 μM AN-treated fungal cultures as well as between the 53 μM DLAeS and 180 μM AN-treated fungal samples. Additionally, the same statistical test indicated that the 28 hr biomass yields (cells/mL) of each treatment were significantly different from each other (Figure 5). Compared to DLAeS, the AN-null DLAeG was ineffective against *C. albicans* and *C. tropicalis* at both 16 and 28 h growth (data not shown).

Single vs. multiple dose treatments

C. albicans cultures with both single and multiple doses of 180 μM AN had less growth than cultures with 52 μM DLAeS at 16 hs growth (Figure 6A,B). At 28 hs, only cultures with multiple doses of AN had a significantly lower percent growth than DLAeS-exposed cultures. At 16 hs, exposing *C. albicans* to a single dose of 52 μM DLAeS reduced fungal growth significantly more than multiple doses of DLAeS. Multiple and single doses of 180 μM AN had a similar effect on growth. However, at 28 hs the effect of multiple and single dose 52 μM DLAeS treatments were not significantly different. In contrast, multiple doses of 180 μM AN reduced growth significantly more than a single 180 μM AN dose at 28 hs of growth (Figure 6B). At 16 h, single doses of both 180 μM AN and 52 μM DLAeS significantly reduced viability (CFUs) relative to the negative control (Figure 6C). By 28 h, there was no statistical significance between the number of CFUs relative to the negative control (Figure 6D). There was also no significant difference between the control and either AN or DLAeS at their respective IC_{50}s, and no statistical difference was observed between AN and DLAeS at their IC_{50}s at either 16 or 28 hs for multiple doses (Figure 6E,F).

Effects of equimolar AN and DLAeS AN at 180 μM AN

Growth and viability (CFUs) were measured to compare the fungicidal capability at 16 and 28 hs of 180 μM AN and 180 μM DLAeS treated *C. albicans* after one dose delivered at 0 h (Figure 7). There was no significant difference between 180 μM DLAeS- and 180 μM AN

treated cultures at both 16 and 28 h (Figure 7A,B). At 16 hs, AN and DLAeS significantly reduced the observed viability of *C. albicans* relative to the 3% DMSO control (Figure 7), but there was no difference between AN and DLAeS at 180 μM (P=0.3266) (Figure 7C). At 28 hs, there was no significant difference in CFUs between any of the samples (Figure 7B,D).

Discussion and conclusions

A. annua and artemisinins vs. Babesia

The parasitemia profile in this study was similar to that observed in other *B. microti*-infected mice (15,38). Although many antimalarial compounds were ineffective against *B. microti* including chloroquine, artesunate, mefloquine and halofantrine (39–43), artesunate did delay the peak of parasitemia during treatment, but it failed to eradicate the parasite. Parasitemia reached 19% and 24% only 5 days after the last treatment of 10 and 50 mg/kg, respectively (15). Animals treated with DLA suffered no adverse effects similar to our prior rodent studies (4,44,45).

Among all *Babesia* species, *B. microti* and *B. equi* are unique parasites and were for a long-time a source of classification debate. *B. microti* lacks schizont replication that is characteristic of other *Babesia* species but uniquely has a transovarian transmission similar to *Theileria*. Using the full sequences of 316 genes to study the phylogeny of Babesiidae, *B. microti* was placed as paraphyletic branch, a result confirming distinctions between *B. microti* and other piroplasms (24). *B. microti* has the smallest genome size among all babesiidae with only three nuclear chromosomes and an overall genome size about 72% less than *P. falciparum* and with a 34% reduction in gene number (24). Unlike malaria, *B. microti* lacks a digestive vacuole, hemazoin formation (46,47) and essential proteases required for hemoglobin digestion (24). The apicoplast function in *B. microti* is limited to production of isoprenoid precursors and lacks *de novo* synthesis of heme and fatty acids (24).

The antimalarial activity of artemisinin depends on heme binding to the peroxide bridge, releasing a carbon free radical that alkylates, modifies and inhibits essential parasite targets (48–50). Heme also enhances artemisinin-SERCA binding and the heme-artemisinin complex interacted with the SERCA pump causing modification in the pump's molecular structure, another active site linked to artemisinin activity (49). Inorganic iron was insignificantly interacting with artemisinin under biological conditions (50). Artemisinin may also be activated by *de novo* synthesized heme produced in the apicoplast and that may explain artemisinin antiparasitic activity against other parasites lacking a digestive vacuole or hemoglobin digestion but known to have de novo synthesis of heam such as *Toxoplasma gondii, Trypanosoma brucei* and probably other species of *Babesia*. The lack of hemoglobin digestion, digestive vacuole, and *de novo* heme synthesis in *B. microti*, may explain the lack of susceptibility to artemisinin, artesunate, and DLA.

One might argue that AN was not bioavailable enough to achieve an adequate serum concentration in the mice. Although poorly bioavailable as a pure compound, when delivered via the plant as DLA, AN is >40 fold more bioavailable than pure AN (18,45,51). Furthermore, the half-life ($t_{1/2}$) of pure AN in mice is about 18.8 min (52), but from the

plant is about 51.6 min (45), indicating that DLA more than doubles the AN half-life in rodents.

A. annua and artemisinins vs. Candida sp

Overall, AN, DLAeS, and some AN derivative drugs had some antifungal effect on *C. albicans, C. glabrata, C. lusitaniae*, and *C. tropicalis.* Almost all of the IC_{50} values determined at mid-log (16 h) for the drugs/extract against each fungal strain were significantly different from each other. AN and DLAeS reduced viability of *C. albicans* at 16 hs growth, suggesting that DLAeS would be equal to or more potent than AN and AN-derivative drugs in inhibiting *Candida* growth. However, by 28 h, the IC_{50} values rose beyond 50 μM indicating they were not effective against *Candida sp*. in their stationary phase. Similar to azoles (53),results showed that artemisinins and *A. annua* were fungistatic and not fungicidal. *Candida sp*. form biofilms , and the lack of activity against cultures in or approaching stationary phase could be due to biofilm formation, which was observed on culture tube walls ≥16 h. Indeed, in a 2019 report by Dominguez *et al.* (54) researchers showed that biofilms produced by *C. auris* sequestered 70% of available triazole drug.

The IC_{50} values in this study were much higher relative to other antifungal drugs, e.g., fluconazole against *C. albicans* was 0.384 μM (55). However, artemisinic drugs and or DLA in future work should be studied in combination with fluconazole and other antifungal drugs as a potential combination therapy. Various phytochemicals in *A. annua* may be synergistic in combination with other antifungal drugs. For example, quercetin had an IC_{50} >0.66 M (56) against *C. albicans* strain SC5314, the same strain used in this study. Despite having a high IC_{50}, a 2016 study found that quercetin works synergistically with fluconazole to target *C. albican* biofilm formation (57). It is thus possible that DLA may have synergistic potential with fluconazole and act as an effective combination therapy against *Candida sp*. Recently, other pharmaceutical formulations reportedly worked synergistically with antifungal drugs including: chlorhexidine, povidone iodine, solutions of gentian violet, potassium permanganate, methylene blue, sodium hyposulfite, propylene glycol, selenium sulphide, boric acid, and caffeic acid derivatives, suggesting that there are more synergistic compounds than previously thought (53).

Multiple doses of AN and DLAeS at their respective IC_{50}s did not significantly alter fungal viability for either 16- or 28 h cultures suggesting that multiple doses of AN and DLAeS are fungistatic but not fungicidal against *C. albicans*. Perhaps use of multiple doses of AN, artemisinin derivatives or DLA would work best to slow the growth of early infections when cells are most metabolically active and growing. The Center for Disease Control recommends multiple doses of antifungals such as fluconazole to treat fungal infections (58).

In summary, results to date do not support substantial efficacy of *Artemisia annua* against either *Babesia microti* or six species of *Candida.*

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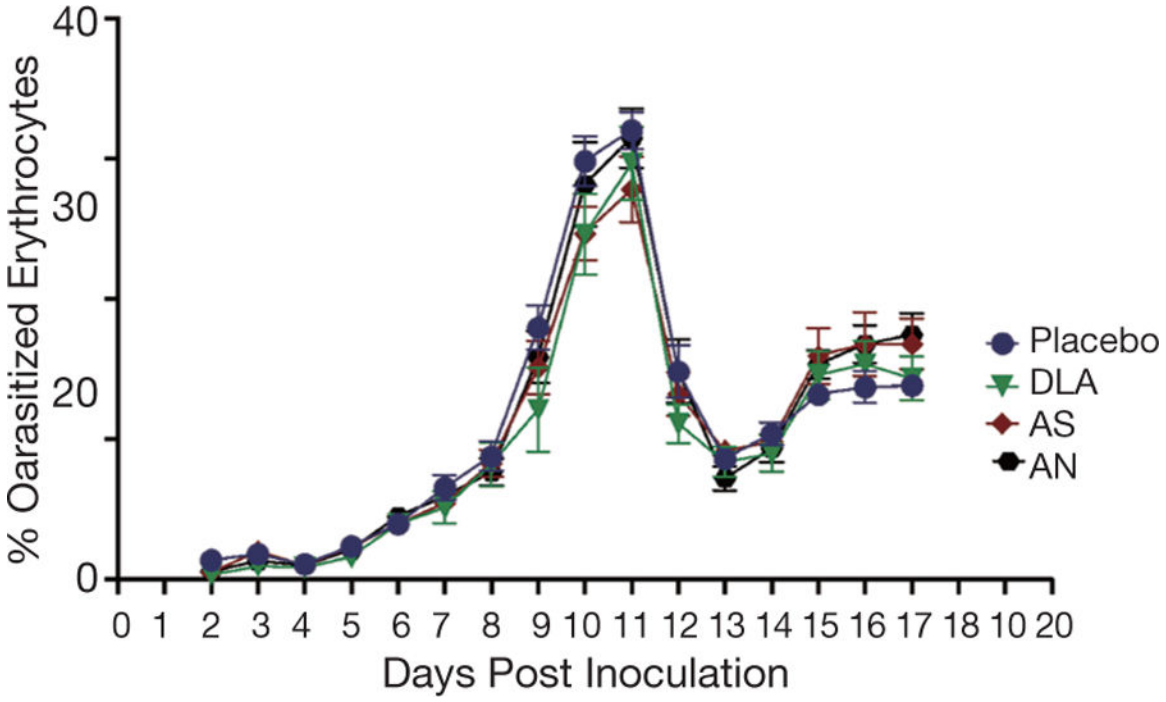

Figure 1.

Babesia microti parasitemia in mice (n=6) post parasite inoculation. Drugs were administered 2 d post infection. AN, 100 mg/kg artemisinin (=167 mg/kg of powdered DLA); DLA, 100 mg/kg of DLA-delivered AN; AS, artesunate 100 mg/kg; placebo is an untreated control fed only mouse chow in a water slurry.

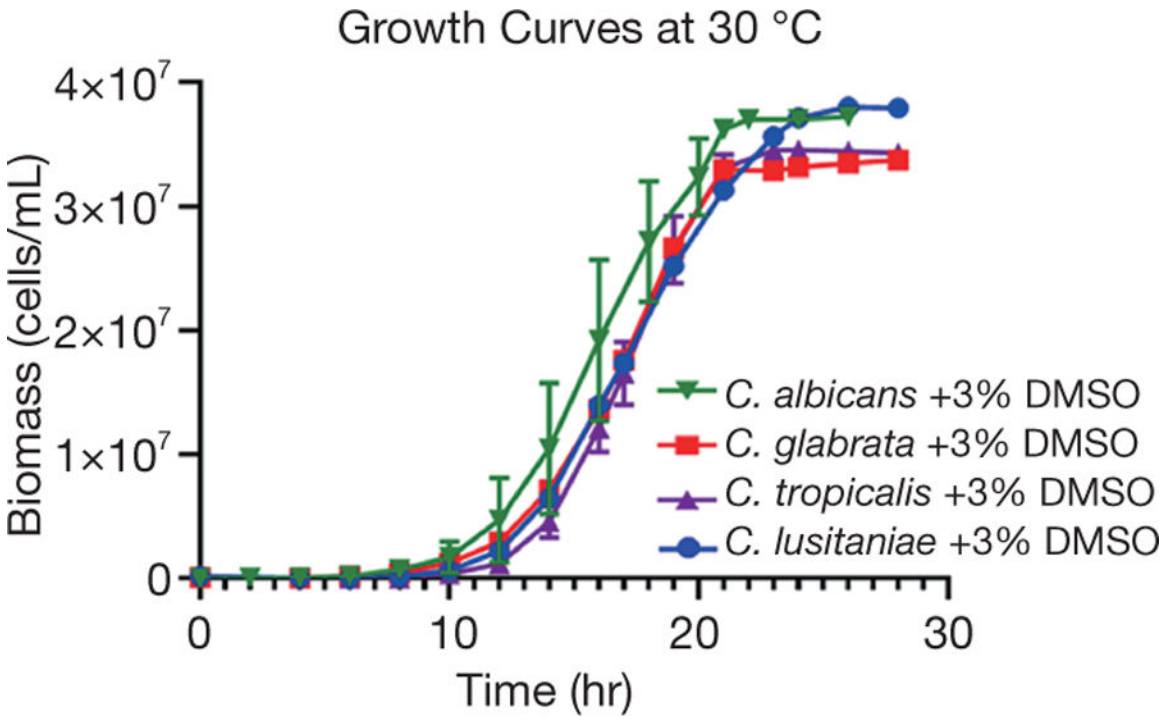

Figure 2.
Candida sp. growth kinetics in the presence of 3% DMSO.

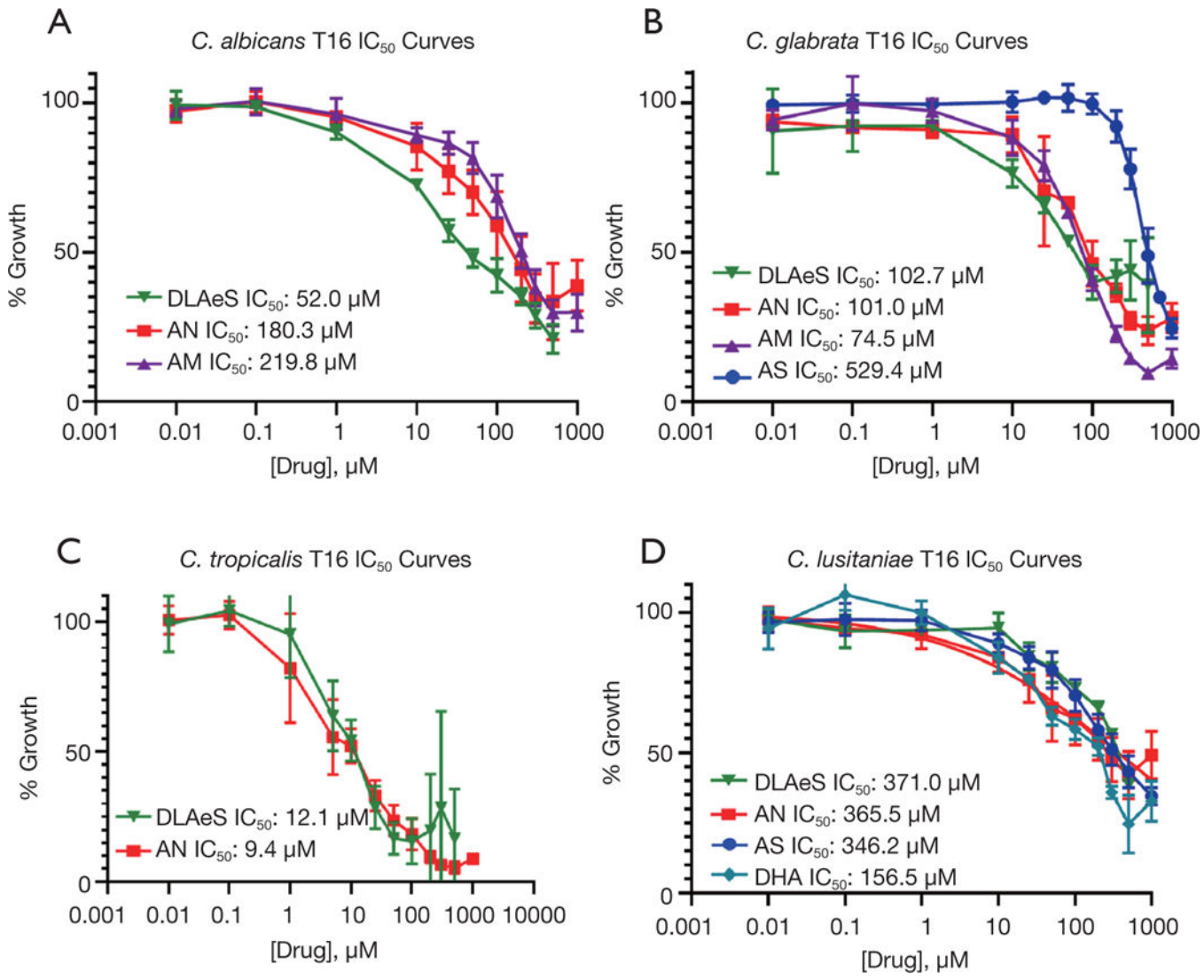

Figure 3.

IC50 curves for Candida sp. at 16 hs growth. *C. albicans* (A), *C. glabrata* (B), *C. tropicalis* (C), and *C. lusitaniae* (D) drug/extract IC50 curves at 16 hs of growth (N=3 for all species except for *C. albicans* AN and AM, which has an N=5). The curve for each species is normalized to the control containing 3% DMSO. ****P<0.0001.

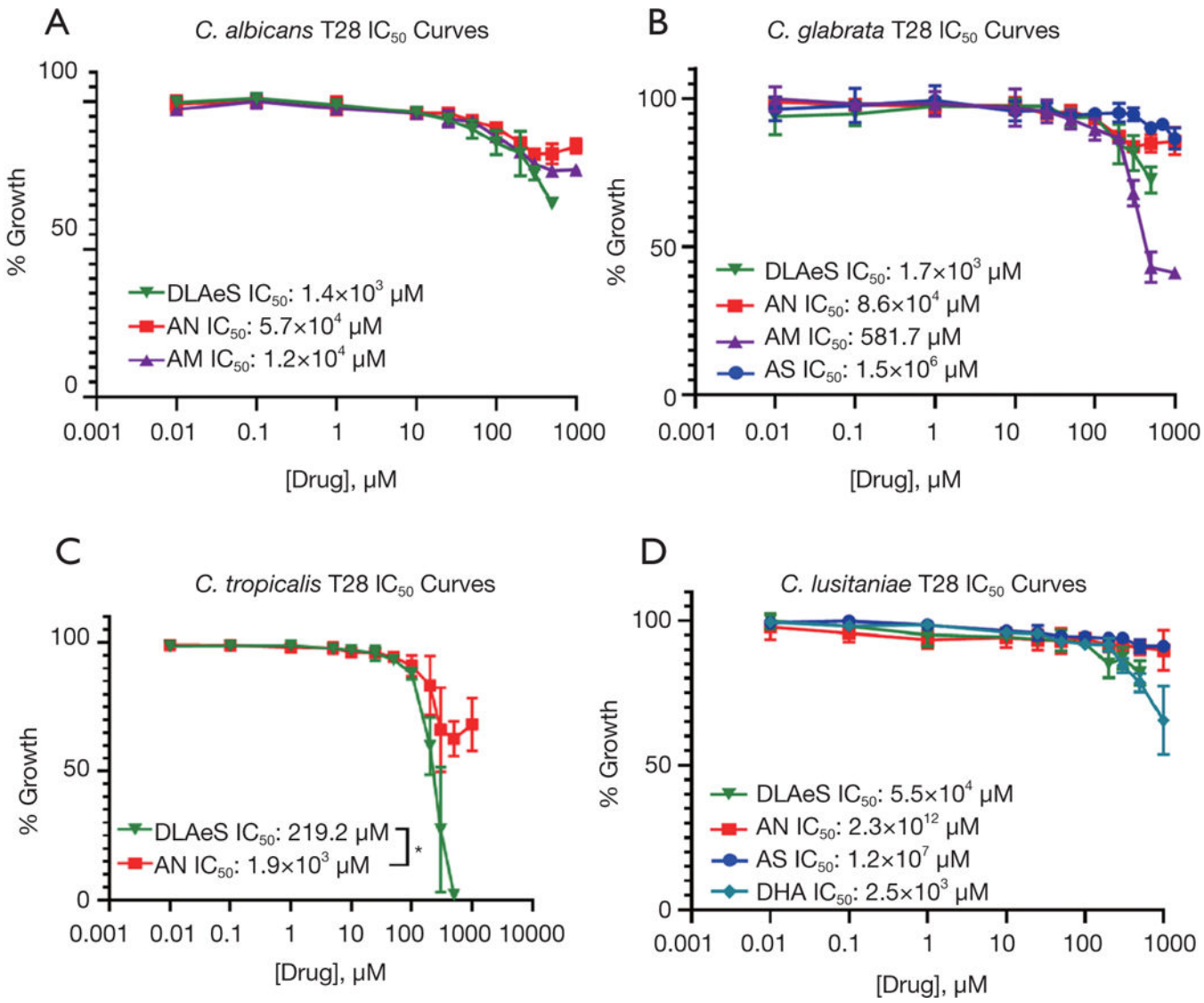

Figure 4.

IC50 curves for Candida sp. at 28 hs growth. *C. albicans* (A), *C. glabrata* (B), *C. tropicalis* (C), and *C. lusitaniae* (D) drug/extract IC50 curves at 28 hs of growth (N=3 for all species except for *C. albicans* AN and AM, which has an N=5). The curve for each species is normalized to an appropriate control containing 3% DMSO. *P<0.5.

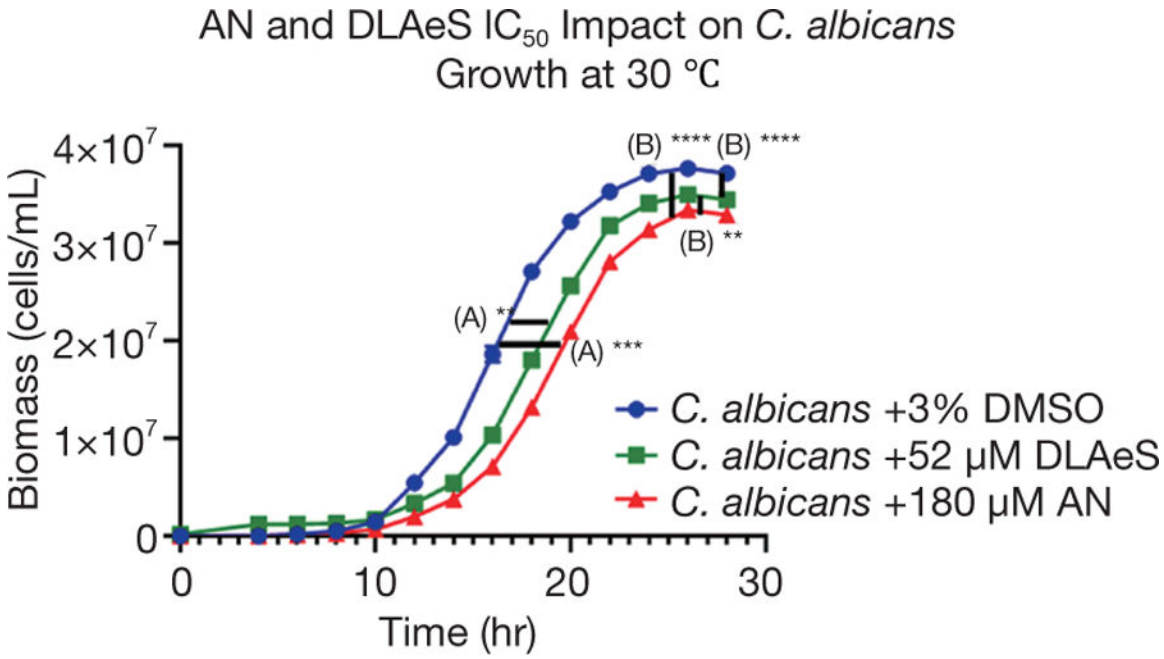

Figure 5.

Growth curves of *C. albicans* in the presence of 3% DMSO, 52 µM DLAeS, or 180 µM AN. **P<0.01, ***P<0.001, ****P<0.0001. (A)Significant difference refers to the comparison between the midpoints of the indicated curves. (B)Significant difference refers to the comparison between the yield of the indicated curves.

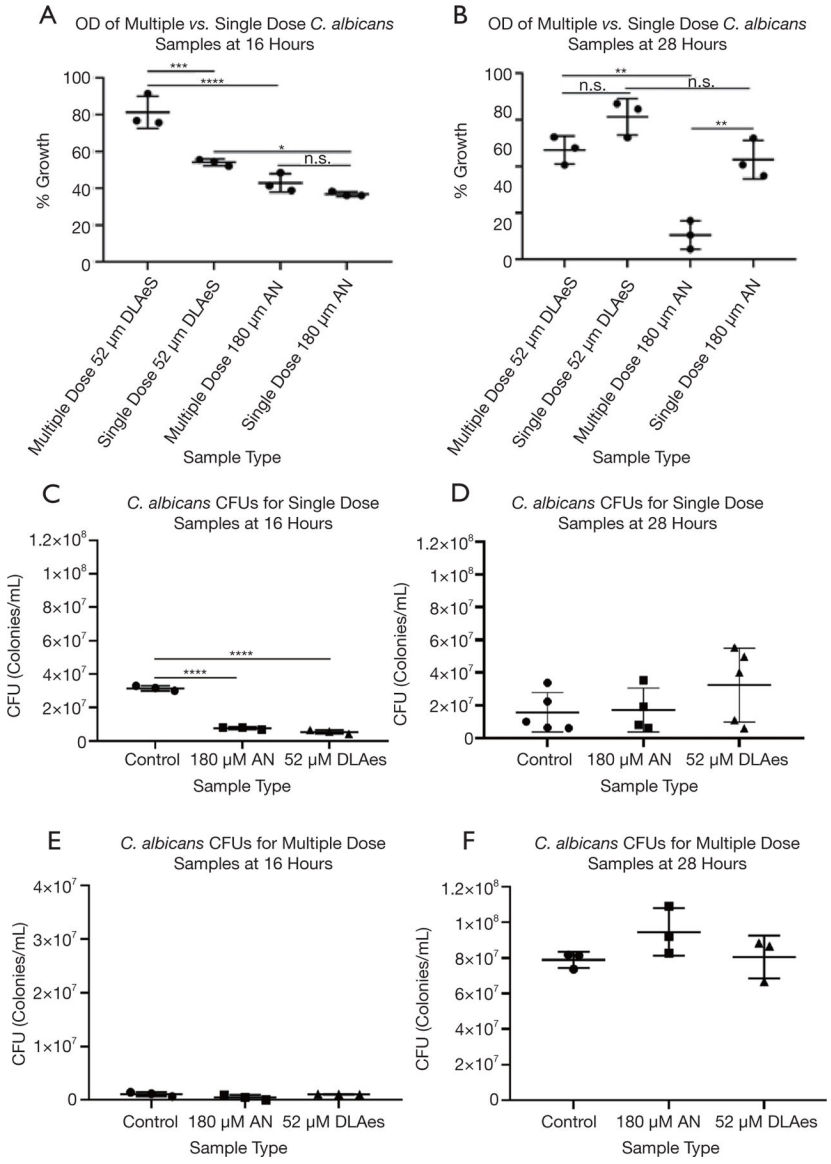

Figure 6.

Single vs. multiple doses of AN or DLAeS *vs. C. albicans* at 16 and 28 hs. (A & B) The growth of *C. albicans* exposed to three doses of 3% DMSO, 180 μM AN, or 52 μM at 0, 6, and 22 h or to a single dose of the same treatments at 0 h is compared. Each graph compares the OD of samples at 16 h (A) or 28 h (B). Percent growth is normalized to 3% DMSO control. (C-F) Column scatter plots for the CFUs observed in single dose (C,D) and multiple dose (E,F) *C. albicans* samples for each tested sample type when grown on YPD media plates for 20 h at 30 ℃. Each graph compares the observed CFUs for the 3% DMSO-exposed control fungal samples relative to 180 μM AN- and 52 μM DLAeS-exposed fungal samples at either 16 h (C and E) or 28 h (D and F) growth. n.s. =no significance, *P<0.05, **P<0.01, ***P<0.001, ****P<0.0001.

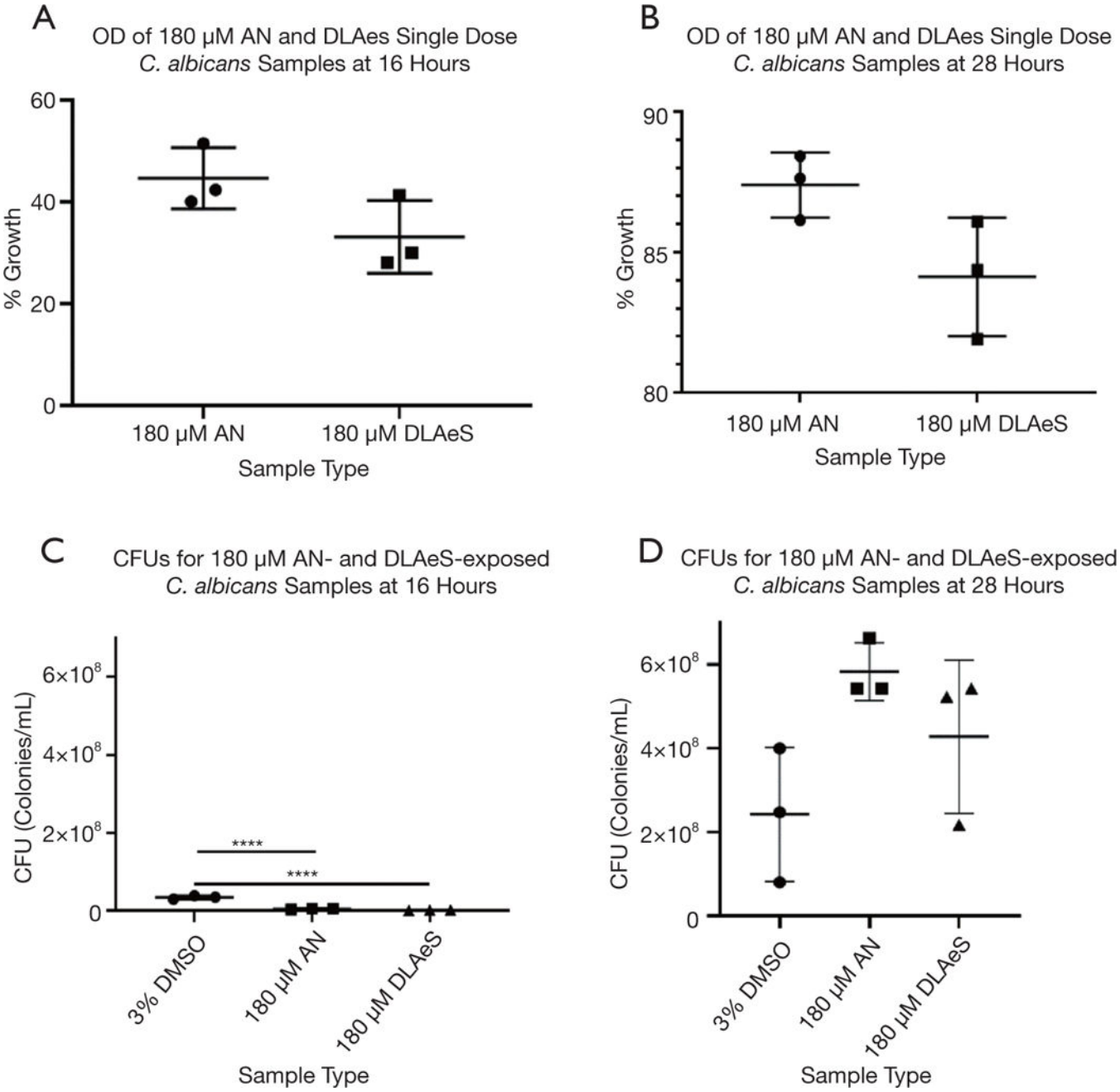

Figure 7.

C. albicans growth with single dose of 180 μM AN or 180 μM DLAeS. (A & B) Percent growth of *C. albicans* when exposed to a single dose of 180 μM AN or 180 μM DLAeS at 0 h normalized to 3% DMSO control. The OD of samples were measured at 16 h (A) and 28 h (B). (C & D) Column scatter plots for the CFUs observed in single dose 3% DMSO, 180 μM AN, and 180 μM DLAeS *C. albicans* samples when grown on YPD media for 20 h at 30 ℃. Each graph compares the observed CFUs for the 3% DMSO-exposed control relative to 180

μM AN- and 180 μM DLAeS-exposed fungal samples as well as compare CFUs for the drug-exposed fungal samples to each other at either 16 h (C) or 28 h (D). ****P<0.0001.

Table 1

Observed zones of inhibition around tested drug-infused filter paper disks for each *Candida* strain after 16 and 20 hs of growth at 30 ˚C

Candida sp.	**Time (hr)**	**Halos present (Y/N)**						
		AN	**DLAeS**	**AS**	**DHA**	**AM**	**DLAeG**	**DMSO**
C. albicans	16	Y	Y	N	N	Y	N	N
	20	Y	Y	N	N	Y	N	N
C. dubliniensis	16	N	Y	Y	N	Y	N	N
	20	N	Y	Y	N	Y	N	N
C. lusitaniae	16	Y	Y	Y	Y	Y[1]	N	N
	20	Y	Y	Y	Y	N	N	N
C. parapsilosis	16	N	N	Y	N	N	N	N
	20	N	N	N	N	N	N	N
C. tropicalis	16	Y	Y	Y[2]	N	Y[2]	N	N
	20	Y	Y	N	N	N	N	N
C. glabrata	16	Y	Y	Y	N	Y	N	N
	20	Y	Y	Y	N	Y	N	N

(I) Halo smaller than for AN, so this drug was excluded from further testing.

(II) Halos were faint at 16 h and not present after 20 h growth, so these drugs excluded from further testing.

Table 2

IC_{50} values for selected Candida species at 16 and 28 hs of growth

Candida sp.	Assay time (hr)	IC_{50}				
		AN (μM)	DLAeS AN (μM) (μg leaf dry mass)	AM (μM)	AS (μM)	DHA (μM)
C. albicans	16	180.9	$52.0^{(1)}$ (42.4 μg DLAeS)	219.8	NM	NM
	28	5.7×10^{4}	$1.4\times10^{3(1)}$ (1.3×10^{5} μg DLAeS)	1.2×10^{4}	NM	NM
C. glabrata	16	101.0	$102.7^{(2)}$ (87.0 μg DLAeS)	74.5	529.4	NM
	28	8.6×10^{4}	$1.7\times10^{3(2)}$ (1.0×10^{5} μg DLAeS)	581.7	1.5×10^{6}	NM
C. tropicalis	16	9.4	$12.1^{(2)}$ (14.3 μg DLAeS)	NM	NM	NM
	28	1.9×10^{3}	$219.2^{(2)}$ (259.9 μg DLAeS)	NM	NM	NM
C. lusitaniae	16	365.5	$371.0^{(2)}$ (314.2 μg DLAeS)	NM	346.2	156.5
	28	2.3×10^{12}	$5.5\times10^{4(2)}$ (3.3×10^{6} μg DLAeS)	NM	1.2×10^{7}	2.5×10^{3}

*(1)*DLAeS biomass is based on a DLAeS plant extract containing 0.91% AN.

*(2)*DLAeS biomass is based on a DLAeS plant extract containing 1.4% AN. NM, not measured because these drugs were previously determined by the halo assay to be ineffective.