**Summary of the Research Excellence**

Dr. Mohammad Owais is a Professor at the IBU, Aligarh Muslim University, Aligarh. Besides active involvement in teaching modern biochemistry/biotechnology courses to M. Sc. students, Dr. Owais has built an active research group with focus on nano-particles-based novel delivery systems such as dendrimers and virosomes for gene packaging and liposomes, niosomes and microspheres for vaccine delivery, gene delivery, targeted drug delivery *etc*; with a view to increase the efficacy and safety of encapsulated chemo-therapeutic agents/sub-unit vaccines for respective diseases.

The research focus of Dr. Owais's group has been on:

1. **Development of nano-particle based antigen/DNA vaccine against various infectious diseases with special converge on intracellular pathogens.**
2. **Development of novel nano-carriers for targeted delivery of encapsulated therapeutic agents (si RNA/drug of interest) for improved treatment of cancer and some imperative infectious diseses.**
3. **Assorted applications of nano-particles in diagnostics, taste/odour masking and treatment of hyperbilirubinemia in model animals.**

(Dr. CM Gupta)

(M. Owais)
Nominee

## 1. Details of the Excellence in Research Work

Dr. Mohammad Owais is currently serving as a professor of biotechnology at Aligarh Muslim University, Aligarh. Besides active involvement in teaching modern biochemistry/biotechnology courses to M.Sc./Ph.D. students, Dr. Owais has successfully established a small but active research group with focus on nano-particle-based novel delivery systems including dendrimers/virosomes for gene packaging and liposomes, niosomes, microspheres and solid core lipid nano-particles for vaccine delivery, gene delivery, targeted drug delivery *etc*; with a view to increase the efficacy and safety of encapsulated chemo-therapeutic agents/sub-unit vaccines for some important infectious diseases.

The research focus of Dr. Owais's group has been on:

- Nanoparticles based antigen/DNA vaccine against various infectious diseases with special converges on intracellular pathogens.

- Novel nano-carriers for targeted delivery of encapsulated therapeutic agents (siRNA/drug of interest) for improved treatment of cancer and some imperative infectious diseases.

- Nanoparticles with assorted applications in the field of diagnostics, taste/odor masking and treatment of hyper-bilirubinemia in model animals.

### *1. Nano-carrier based vaccines: prophylactic measures against major infectious diseases.*

Reckoning with the limitations of conventional vaccines, the main focus of Dr. Owais's research endeavors has been to develop nano-vaccines against various infectious diseases of bacterial (tuberculosis, salmonellosis, listeriosis and brucellosis), protozoan (malaria, leishmaniasis) and fungal (candidasis and cryoptococcosis) origin.

In general, specialized groups of pathogens adapt intracellular parasitism as a strategy to avoid antibody onslaught. Keeping into consideration the non-effectiveness of humoral immune response against intra-cellular pathogens, Dr. Owais evaluated potential of amyloid fibril based vaccines against various intracellular pathogens such as *M. tuberculosis* and *Brucella abortifaciens, etc*. (Saba *et. al* JBC 2014, Faraz et al., Frontiers in Immunology and Tufail et. al. JBC 2018). Next, he assessed prophylactic potential of fusogenic lipid based vaccines as an alternative prophylactic strategy. In this regard, he has compared lipid compositions of plasma membranes of both prokaryotic as well as eukaryotic cells. These studies established a correlation between the lipid compositions of plasma membranes of living organisms with evolutionary trend (Deba *et. al*. BBA 2005). Lipid isolated from lower organisms possesses strong fusogenic potential (Owais *et. al*. FEBS J 1999, Ahmad *et. al*. FEBS J 2000, Farah *et. al*. BBA 2005, Ansari *et. al*. Plos One 2011). He further established that model antigens entrapped in liposomes made up of fusogenic lipids can be delivered to the target cells including antigen presenting cells. This eventually facilitates both endo/lysosomal and cytosolic degradation pathways for antigen processing. The dual processing of antigens in the antigen presenting target cells activated both the CD4+ T helper as well as CD8+ T cytotoxic cells. Further, he established that immunization with fusogenic liposomes resulted in expression of both IL-2 and IFN-γ, the two key cytokines that eventually help in protection against intracellular infections (Faisal *et. Al* Vaccine 2003, Farah *et. al*, BBA 2005, Atif *et. al*, FEBS Letters 2006, Sharad *et. al*, Vaccine 2006).

Keeping in view that sperm-ova fusion during zygote formation is generally facilitated by specific lipid compositions of the two cell populations, he demonstrated the fusogenic attributes of sperm plasma membrane lipids (Atif *et. al*. Vaccine 2008) and established the prophylactic potential of spermatsome based vaccines against various intracellular pathogens (Atif *et. al*. Vaccine 2010).

As conventional egg phosphatidyl-choline (PC) based liposomes are of limited application in activation of pathogen specific CTL response required for inhibiting intra-cellular pathogens, Dr. Owais developed non-PC liposome as vehicle for delivery of antigens in prophylactic treatment of experimental leishmaniasis (Sharad *et. al*. Vaccine 2006). Further, the liposome/niosome based vaccines were also found to be effective against malaria parasite (Sharma *et. al*. Vaccine 2006, Sharma *et. al*. Vaccine, 2007, Varun *et. al;* Pharmaceutical Research 2009). In addition, he has prepared Archae lipid based (Archaeosome) liposomes

and demonstrated their immunoadjuvant potential in model animals. Of note the archaeosome based vaccine were used to mount long lasting memory response against experimental listeriosis (Ansari *et. al*. I. J Nanomedicine 2012).

Further, Dr. Owais has highlighted interactions between two mycobacterial proteins viz. Rv3619 (RD9 family) and Rv3620 (CFP-10 analog). He demonstrated that Rv3619 protein disrupted the biomembrane and also evoked a strong immunological response (Mahmood *et. al.* FEBS J 2010). Moreover, it was revealed that nano-particle mediated targeting of RD9 gene products to dendritic cells favors Th1 prototype of CD4+ T lymphocytes. Targeted delivery of encapsulated antigen to dendritic cells was achieved by coupling anti-DEC antibodies to the surface of archaeosome (archaebacteria lipid vesicles), which helped to cut down the antigen dose significantly thereby making the immunization protocol cost effective (Ansari *et. al*. 2011).

He had successfully expressed L7/L12 ribosomal protein, SOD-IL-18 fusion protein of *Brucella sp*. and trypanothione-reductase of *Leishmania donavani*. The recombinant proteins were used as potent sub unit vaccines in protection studies (Sharad*et. al*. Vaccine 2006, Mallick *et. al* Vaccine 2007, Mallick*et. al*. Vaccine 2008). A liposome-based DNA vaccine developed by Dr. Owais has shown remarkable promise against experimental murine brucellosis (Singha*et. al*. Microbes & Infection 2008).

Besides introducing liposome, niosome and microsphere based novel particulate vaccines; Dr. Owais has recently employed an autologous plasma bead based dual antigen delivery system as a prophylactic strategy against intracellular infections (Ejaj *et. al*. Vaccine 2011). The liposome/microsphere entrapped antigen further co-entrapped in dual core fibrin beads based vaccine was shown to eliminate intracellular pathogens from systemic circulation (Khan *et. al.* JAC 2012).

2. ***Targeted nano-delivery system***.

Targeted delivery of anticancer agents and antibiotics has been considered as one of the most coveted endeavors in the field of nano-vehicle based drug delivery technology

employing adjunctive antimicrobial agents. Efforts from Dr. Owais research group to use a combination of nano-particles based formulations with immunomodulators have been highly successful in combating infectious diseases in experimental diseases (Khan *et. al*. FEMS Micro & Immunol.2003, Khan *et. al*. JDT 2004, Khan *et. al*. JAC 2004). His studies suggest that drug delivery potential of nano-particles can be increased considerably by co-entrapment of potential immunomodulators, such as picroliv, tetrapeptidetuftsin, protein A and various analogs of muramyl peptide, *etc*, in combination with the anti-microbial agents. The resulting formulations were found to be effective against treatment of a range of infectious diseases such as fungal (candidiasis, cryptococcosis, aspergillosis), bacterial (tuberculosis, leprosy, salmonellosis), protozoal (leishmaniasis, malaria) nematodes (filariasis) *etc* (Owais*et. al.* FEBS Letters 1993, Owais *et. al.* AAC 1995, Owais *et. al.* FEBS J 1999, Khan *et. al.* JACS 2002, Khan, *et. al.* FEMS Microb. & Immun. 2003, Deeba *et. al.* Biochimie 2005, Sharma, *et. al*. Vaccine 2006, Sharad, *et. al.*Vaccine 2006).

Liposomes have been widely considered useful as drug/enzyme/nucleic acid vehicles in therapy. However, their successful application was limited by their rapid lysis in blood, major uptake by the RES, and lack of availability of simple procedures for specific targeted delivery. The main emphasis of Dr Owais has been therefore on addressing some of the problems associated with the liposomes as drug delivery systems. He demonstrated that covalent attachment of anti-erythrocyte $F(ab')_2$ to the liposomes surface enables the liposomes to specifically recognize the erythrocytes in vivo and deliver their contents to these cells. It was further demonstrated that the entrapment of anti-malarial drugs like chloroquine (chq), in the antibody-coated liposomes increases the drug efficacy not only against the chq-sensitive but also against the chq-resistant malarial infections. Encouraged by these results, the liposomes were coated with $F(ab')_2$ fragments of a monoclonal antibody which specifically recognized the malaria-infected erythrocytes (Patent No. 182550). The monoclonal antibody bearing liposomes with encapsulated chq were found to be highly effective in the treatment of chq-resistant experimental malaria (Owais *et. al.* AAC 1995).

**RNA interference** is a newly discovered cellular mechanism for silencing genes in a sequence specific manner at the mRNA level. It involves introduction of cognate double stranded small interfering RNA (siRNA) to target desired mRNA and has been shown to have

application in viral and cancer therapy. Administration of naked siRNA is susceptible to rapid degradation by plasma RNAses. Cationic lipids have been used as carrier of siRNA, however, not desirable due to innate toxicity of the RNA-lipid complex. To overcome this problem, Dr. Owais has developed a novel nano-particle based formulation encapsulating siRNA that down-regulates Polo like kinase 1(Plk1) and Fox O protein in treatment of skin, liver and breast cancer (Chauhan *et. al.* Nanomedicine 2014, Sherwani et. al. RSC Advances, 2015, Asif et. al. RSC Advances 2015).

Further, the nano-particle based formulations (*cf*. dendrimers, niosomes, liposomes and microspheres) of some important essential oils, viz. clove oil, perillyl alcohol, eugenol and various allyl-sulphide analogs, were first time developed by nominee's group and shown to be effective against drug resistant isolates of various fungal as well as bacterial pathogens (Arif *et. al.* Mol Medicine 2007, Arif, *et. al.* Mol Medicine 2009). Interestingly besides infectious diseases, the pH sensitive as well as fusogenic liposomes-based formulations of diallyl-sulphide were shown to be effective against skin carcinoma in model animals as well (Maroof *et. al*, Nanomedicine 2010, Khan *et. al.* JACS 2011).

As evident from one of his studies that introduction of HIV-1 genome into PBMCs blocks the propagation of HIV-2 viruses, he developed gene therapy vector for transfecting HIV-2 infected PBMCs with HIV-1 genome using SCID mice (Al-Harathy *et. al*. AIDS RHV 1998). Several studies have defined a close relationship between the HIV-1 infection and the components of the immune system involving chemokines. Suppression of HIV by chemokines represents a special case in virology and immunology where soluble molecules other than antibodies inhibit infection by a specific virus. Consequently, studies by Dr. Owais have focused on the role of various domains of chemokines that are responsible for anti-HIV activity or help in inflammatory responses in the host. He cloned genes of important β chemokines such as RANTES and MIP-1α and expressed them in eukaryotic (HEK 293) and insect cells (SF-9 and SF-21). In order to develop chemokine as a future therapeutic agent against the treatment of HIV infection, it is necessary to establish their structure and function relationship. In this context, he successfully characterized the functional domains of β chemokine RANTES in relation to its anti-HIV activity (Owais & Arya J Hum Virol 1998).

### *3. Other applications of novel nano-particles*

a) The research group of Dr. Owais group developed a liposome based mouthwash containing essential oil that binds to the mucus membrane inside the mouth. This enables the essential oils to remain in the mouth for extended time period to achieve long-lasting germ-killing and breath-freshening protection. Because liposomes have a tremendous amount of surface area with which to transfer the essential oils to the mucus membrane, they can magnify the effect of the oils. The encapsulation of the essential oils also protects them from hydrolysis or oxidation (Ahmad *et. al*. JDT, 2004).

b) Bilirubin, a metabolic by-product of hemoglobin, has been considered as an effective biomarker of liver function. The elevated plasma level of bilirubin exerts deleterious effects on the liver function. The liposome/microsphere based nano-carriers developed by Dr. Owais have been found to be potential scavenger of bilirubin from experimental animals (Masood *et. al.* FEMS Micro & Immunol. 2004, Ahmad *et. al*. BBA 2004, Ahmad *et. al*. BBA 2006).

c) Nano-particles have been exploited as an effective tool for diagnostics in detection of cancer as well as the presence of pathogens in various food products. The gold-nano-particle based immunodiagnostic device developed by Dr. Owais has been found to be very effective in cancer diagnosis (Arun *et. al*. IJ Nanomedicine 2011). Besides, aptamer/antibody based biosensor devices developed by Dr. Owais have wide application in detection of food borne pathogens in meat and shrimps industry (Owais *et. al.* Plos One 2014).

## ANNEXURE I

PUBLICATIONS

**i)** Original research papers published in full:

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1. Ahmar RM, Swaleha Z, Hira A, Subodh P, Ajmal KM, **Owais M** (2018) Synergistic effect of Diallylsulphide with Zinc oxide Nanorods: A novel and effective approach for treatment of acute dermatitis in model animals. **Frontiers in Microbiology** 9:586. doi: 10.3389/fmicb.2018.00586 **[IF 4.1]**

2. Tufail S, Sherwani MA, Shoaib S, Azmi S, **Owais M**, Islam N. (2018) Ovalbumin self-assembles into amyloid nanosheets that elicit immune responses and facilitate sustained drug release. **J Biol Chem**: 293(29):11310-11324. doi: 10.1074/jbc.RA118.002550. [Epub ahead of print] **[IF 4.1]**

3. Mirza S, Zia I, Jolly R, Kazmi S, Owais M, Shakir M. (2018) Synergistic combination of natural bioadhesive bael fruit gum and chitosan/nano-hydroxyapatite: A ternary bioactive nanohybrid for bone tissue engineering. **Int J Biol Macromol** 119: 215-224. **[IF 3.7]**

4. Shakir, M., Reshma J., Aijaz Ahmad Khan, Sharique Alam Mohd Shoeb Khan, Mohd. Ahmar Rauf, **Owais, M.,** Mohd. Ahmadullah Farooqui. (2018) Resol based Chitosan/nano-hydroxyapetitenanosensamble for effective bone tissue engineering. **Carbohydr Polym**. 179: 317-327. doi: 10.1016/j.carbpol.2017.09.103. Epub 2017 Oct 3]. **[IF 4.8]**

5. Kaushik S, Iqbal N, Singh N, Sikarwar JS, Singh PK, Sharma P, Kaur P, Sharma S, **Owais M**, Singh TP (2018) Search of multiple hot spots on the surface of peptidyl-tRNA hydrolase: structural, binding and antibacterial studies. **Biochem J**. 475(3): 547-560. doi: 10.1042/BCJ20170666. **[IF 4.4]**
6. Ahmad F, Zubair, S, Gupta P, Gupta UD, Patel R, **Owais M**. (2017) Evaluation of Aggregated Ag85B Antigen for Its Biophysical Properties, Immunogenicity, and

Vaccination Potential in a Murine Model of Tuberculosis Infection. **Front Immunol**. 8:1608. doi: 10.3389/fimmu.2017.01608. **[IF 6.70]**

7. Shoeb, M., Mobin, M., Rauf, A; **Owais, M.,** Naqvi, A. (2018) In vitro and in vivo antimicrobial evaluation of Graphene-Polyindole (Gr@PIn) Nanocomposite against Methicillin Resistant *Staphylococcus aureus* pathogen. ACS Omega 3 (8): 9431-9440.

8. Tauqir, A., Ahmar RM, **Owais, M.,** Abgeena, N. (2018) Green synthesis of silver nanoparticles, its characterization, and chaperone-like activity in the aggregation inhibition of α-Chymotrypsinogen A. Journal of Luminescence. Communication ID: LUMIN_2018_421].

9. Tauqir, A., Ahmar RM, Asim, R., **Owais, M.,** Abgeena N. (2018) Thermal unfolding of human lysozyme induces aggregation: Recognition of the aggregates by antisera against the native protein. **International Journal of Biological Macromolecules** 113, 976-982**. [IF 3.7]**

10. Zia Q, Azhar A, Ahmad S, Afsar M, Hasan Z, **Owais M**, Alam M, Akbar S, Ganash M, Ashraf GM, Zubair S, Aliev G. (2017) PeMtb: A Database of MHC Antigenic Peptide of Mycobacterium tuberculosis. **Curr Pharm Biotechnol.** 10;18(8):648-652. doi: 10.2174/1389201018666170914150115. **[IF 1.6]**

11. Fatima N, Faisal SM, Zubair S, Siddiqui SS, Moin S, **Owais M**. (2017) Emerging role of Interleukins IL-23/IL-17 axis and biochemical markers in the pathogenesis of Type 2 Diabetes: Association with age and gender in human subjects. **Int J Biol Macromol**. 105 (Pt 1): 1279-1288. doi: 10.1016/j.ijbiomac.2017.07.155. **[IF 3.7]**

12. Shakir M, Hanif S, Sherwani MA, **Owais M**, Azam M, Al-Resayes SI. (2016) Pharmacophore hybrid approach of new modulated bis-diimine Cu (II)/Zn (II) complexes based on 5-chloro Isatin Schiff base derivatives: Synthesis, spectral studies and comparative biological assessment. **J Photochem Photobiol B.** 157:39-56. doi: 10.1016/j.jphotobiol.2016.01.019. **[IF 3.2]**

13. Shakir, M., Nausheen, B., Mohd. Ahmar Rauf, **Mohammad Owais.** Pharmacologically significant tetrazaa macrocyclic metal complexes derived from isatin and 3,4-diaminobenzophenone: Synthesis, spectral studies and comparative

invitro biological assessment. **Journal of Chemical Sciences** 129 (12), 1905-1920. **[IF 1.2]**

14. Anam, A., Ali, A., Asif, M., Ahmar RM, **Mohammad Owais, M.** (2018) Facile one-pot multicomponent synthesis of steroidal oxazole/thiazole derivatives with effective antimicrobial, antibiofilm and hemolytic properties. **Steroids** 134, 22-36**. [I.F 2.8].**
15. Shakir, M., Nausheen, B., Ahmar RM, **Owais, M. (2018)** Pharmacological approach for bio-relevant N-functionalized homo-binuclear macrocyclic complexes based on 16 membered tetraaza units: Synthesis, spectral studies, biological screening, HSA binding and molecular docking. **[**Accepted in Journal of Inclusion Phenomena and Macrocyclic Chemistry].
16. Qamar Zia, Mohd. Ahmar Rauf, Wasi Ahmad **Owais M.** Biomimetically engineered Amphotericin B nano-aggregates circumvent toxicity constraints against mammalian cells. **Scientific reports** 7 (1), 11873. **[IF 4.3]**
17. Tan, D., Zia, Q., Zubair, S., Stapleton, P., Singh, R., Owais, M., Somavarapu, S. (2017) B Novel biodegradable poly (gamma-glutamic acid)-amphotericin B complexes show promise as improved amphotericin B formulations. Nanomedicine: Nanotechnology, Biology, and Medicine (Elsevier) pii: S1549-9634(17)30021-7. doi: 10.1016/j.nano.2017.02.003. **(Impact Factor: 6.70)**
18. Ahmar, RM, **Owais, M.,** Ravikant, R., Faraz, A., Nazoora, K., Swaleha Z. (2017) Biomimetically synthesized ZnO nanoparticles attain potent antibacterial activity against less susceptible *S. aureus* skin infection in experimental animals. *RSC Adv.*, 7: 36361-36373. **[IF 3.2]**
19. Shakir, S. Mirza, R. Jolly, A. Rauf and **M. Owais,** (2017) Synthesis, Characterization and in vitro screening of nano Hydroxyapatite/Chitosan/Euryale ferox Nano ensemble- An inimitable approach for Bone Tissue Engineering. M. New Journal of Chemistry 42 (1), 363-371. [IF 3.2]
20. Shakir, M., Nausheen, B., Ahmar RM, **Owais, M. (2017)** Pharmacological approach for bio-relevant N-functionalized homo-binuclear macrocyclic complexes based on 16 membered tetraaza units: Synthesis, spectral studies, biological

screening, HSA binding and molecular docking. **[**Accepted in Journal of Inclusion Phenomena and Macrocyclic Chemistry].

21. Laskar, K., Mohd, SF., Abdul, R., Anees, A., Owais, M. (2017) Undec-10-enoic acid functionalized chitosan based novel nano-conjugate: an enhanced anti-bacterial/biofilm and anti-cancer potential Carbohydrate Polymers. 166: 14–23.(Impact Factor: 4.10)
22. Zia Q, Azhar A, Ahmad S, Afsar M, Hasan Z, Owais M, Alam M, Akbar S, Ganash M, Ashraf GM, Zubair S, Aliev G. (2017) PeMtb: a database of MHC antigenic peptide of Mycobacterium tuberculosis. Curr Pharm Biotechnol.doi: 10.2174/1389201018666170914150115. [Epub ahead of print] (Impact Factor: 2.40)
23. Zubair S, Azhar A, Khan N, Ahmad E, Ajmal M, Owais M. (2017) Nanoparticle-Based Mycosis Vaccine. Methods Mol Biol. 1625:169-211. doi: 10.1007/978-1-4939-7104-6_13. (Impact Factor: 3.80) [Points: 12/6= 2.0]
24. Owais M, Kaur J, Singh G, Faisal SM, Azhar A, Rauf MA, Gupta UD, Gupta P, Pal R, Zubair S. (2016) TLR Agonist Augments Prophylactic Potential of Acid Inducible Antigen Rv3203 against Mycobacterium tuberculosis H37Rv in Experimental Animals. PLoS One. 29;11(3): e0152240. (Impact Factor: 3.45)
25. Fatima N, Faisal SM, Zubair S, Ajmal M, Siddiqui SS, Moin S, Owais M. (2016) Role of Pro-Inflammatory Cytokines and Biochemical Markers in the Pathogenesis of Type 1 Diabetes: Correlation with Age and Glycemic Condition in Diabetic Human Subjects. **PLoS One**. 2016 Aug 30;11(8): e0161548. doi: 10.1371/journal.pone.0161548 (Impact Factor: 3.45).
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27. Zafar H, Kareem A, Sherwani A, **Owais M**, Ansari MA, Khan HM, Khan TA. (2015) Synthesis and characterization of Schiff base octaazamacrocyclic complexes and

their biological studies. **J Photochem Photobiol B.** 142:8-19. doi: 10.1016/j.jphotobiol.2014.10.004. Epub 2014 Oct 24.

28. Asif, MS, Tufail, S., Khan, AA, Owais, M. (2016) Dendrosome mediated topical gene silencing by PLK-1 specific siRNA: implication in treatment of skin cancer in mouse model. RSC Advances 6 (8), 6843-6857 (Impact Factor: 3.84)
29. Zaheer MR, Gupta A, Iqbal J, Zia Q, Ahmad A, Roohi, Owais M, Hashlamon A, Mohd Setapar SH, Ashraf GM, Aliev G. (2016) Molecular mechanisms of drug photodegradation and photosensitization. Curr Pharm Des.; 22(7):768-82. (Impact Factor: 2.84)
30. Azhar A, Ahmad E, Zia Q, Owais M, Ashraf GM. (2016) Recent updates on molecular genetic engineering approaches and applications of human therapeutic proteins. Curr. Protein Pept Sci. 2016. [Epub ahead of print]
31. Hassan MF, Rauf A, Sherwani A, Owais M. (2015) Synthesis and In Vitro Biological Evaluation of 1,3,4-Oxadiazol-2(3H)-one and Tetrahydropyridazine-3,6-dione Derivatives of Fatty Acids. Sci Pharm. 9;83 (3):429-43.
32. Ahmad E, Zia Q, Fatima MT, **Owais M**, Saleemuddin M (2015) Vaccine potential of plasma bead-based dual antigen delivery system against experimental murine candidiasis.Int J Biol Macromol. 81:100-111. (Impact Factor: 2.65)
33. Ahmad E, Fatima MT, Hoque M, Owais M, Saleemuddin M. (2015) Fibrin matrices: The versatile therapeutic delivery systems. Int J Biol Macromol. 81:121-136. (Impact Factor: 2.65)
34. Sherwani MA, Tufail S, Khan AA, **Owais M**. (2015) Gold Nanoparticle-Photosensitizer Conjugate Based Photodynamic Inactivation of Biofilm Producing Cells: Potential for Treatment of C. albicans Infection in BALB/c Mice. **PLoS One**. 2015 Jul 6;10 (7): e0131684. doi: 10.1371/journal.pone.0131684. (Impact Factor: 3.45)
35. Tufail S, Badrealam KF, Sherwani A, Gupta UD, Owais M. (2013) Tissue specific heterogeneity in effector immune cell response. Frontiers in Immunology4:254. doi: 10.3389/fimmu.2013.00254.
36. Sherwani, M. A., Tufail, S., Khan A. A, Owais, M. (2015) Dendrimer-PLGA based multifunctional immuno-nanocomposite mediated synchronous and tumor

selective delivery of siRNA and cisplatin: potential in treatment of hepatocellular carcinoma **RSC Advances** 5 (49): 39512-39531(Impact Factor: 3.88)

37. Ansari, MA, Qamar, Zia, Khan AA, Azhar, A, Owais, M. (2015), Efficacy of cell wall deficient spheroplasts against experimental murine listeriosis. **Scandinavian Journal of Immunology** 82(1):10-24. (Impact Factor 2.15)
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48. Ahmad A, Varshney H, Rauf A, Sherwani A, Owais M (2014). Synthesis and anticancer activity of long chain substituted 1, 3, 4-oxadiazol-2-thione, 1, 2, 4-triazol-3-thione and 1, 2, 4-triazolo [3, 4-b]-1, 3, 4-thiadiazine derivatives. Arabian Journal of Chemistry doi:10.1016/j.arabjc.2014.01.015 (Impact Factor: 3.7)
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***iii)*** *Scientific Reviews:*

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i) **Other publications (poster presentation)**:

- Paper entitled as “Liposome in treatment of infectious diseases” was presented in Second Chandigarh Symposium on “New Biology” at IMTECH, Chandigarh during March 22-23, 1993.
- AIDSLINE ICA12/98385118. Meeting Jan 1998. National Cancer Institute, National Institute of Health, Bethesda, MD, USA. Anti-HIV chemokines: domain mapping and HIV-2 lentivirus delivery.
- International Conference on “Current Trends in Drug Discovery Research (CTDDR)” at CDRI, Lucknow during Feb 13-17 2001 and presented poster entitled “Liposome mediated removal of bilirubin in jaundice rats.”
- 9th Asia Pacific Congress in Clinical Biochemistry, 2002 at New Delhi during March 9-14, 2002and presented a poster entitled “Binding of bilirubin with albumin coupled liposomes: Implication in treatment of jaundice.”
- Yeast 2003: An International meeting on yeast biology at IMTECH, Chandigarh during Feb 20-22, 2003 and presented poster entitled “Reconstitution of Candida albicans antigen in fusogenic yeast lipid vesicles: Implication in vaccine development.”
- Yeast 2003: An International meeting on yeast biology at IMTECH, Chandigarh during Feb 20-22, 2003 and presented poster entitled “Glyoxylate cycle enzymes as potential drug targets for treatment of intracellular infections.”
- 2nd World Congress on “Biotechnological developments of herbal medicines” at NBRI Lucknow during Feb 20-22, 2003, and presented poster entitled “Antibacterial efficacy of Withania somnifera against experimental Salmonella typhimurium infection in BALB/c mice.”
- 6th International Conference on “Liposome Advances: Progress in drug and vaccine delivery” at School of Pharmacy, University of London, London, UK during Dec 15-19, 2003, and presented a poster entitled “Fusogenic liposomes: potential as future vaccine candidates.”

- Indo-Australian Conference on Biotechnology in infectious diseases at Kasturba Medical College, MAHE, Manipal during 1-3 March, 2005, and presented poster entitled “Role of vaccine adjuvant against experimental murine Salmonellosis.”
- Indo-Australian Conference on Biotechnology in infectious diseases at Kasturba Medical College, MAHE, Manipal during 1-3 March, 2005, and awarded best poster

entitled “Escheriosome entrapped soluble blood stage antigens impart protective immunity against a multidrug resistant isolate of Plasmodium yoeliinigeriensis in BALB/c mice.”

- National symposium on Nano particles, IVRI, Izat Nagar during 22-23 Dec, 2007, delivered talk on Development of nano particle based drug and antigen delivery system.
- International symposium on the Predictive, Preventive and Mechanistic Mutagenesis & XXXIII EMSI annual Meeting, AMU, Aligarh during Jan 1-3, 2008 and presented poster entitled as “Fibrin mesh encapsulated tuftsin activates immune functions of host macrophages.
- International symposium on the Predictive, Preventive and Mechanistic Mutagenesis & XXXIII EMSI annual Meeting, AMU, Aligarh during Jan 1-3, 2008 and presented poster entitled “Fusogenic potential of sperm membrane lipids: nature’s wisdom to accomplish targeted gene delivery.”

## ANNEXURE II

## BOOKs:

1. Modern Phytomedicine: Turning Medicinal Plants into Drugs (2006) Wiley VCH, Verlag Gmbtt & Co. KgaA.
2. Trypanothione reductase: a potential anti-leishmanial drug target (2009) (ISBN-NR 978-3-639-21244-0) VDM Verlag Dr. Müller Aktiengesellschaft & Co. KG
3. Combating Fungal Infections: Problems and Remedy (2010) Springer-Verlag, Heidelberg, Germany.

a) BOOK CHAPTERS/REVIEWS:

1. Herbal Medicines: Prospects and Constraints. Iqbal Ahmad, Aqil F, Ahmad F, Owais M. In: Modern Phytomedicine: Turning Medicinal Plants into Drugs. (2006) Wiley VCH, Verlag Gmbtt & Co. KGaA. pp: 59-76.
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BASIC SCIENCE

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Research Article

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# Development, characterization and efficacy of niosomal diallyl disulfide in treatment of disseminated murine candidiasis

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**Abstract**

In the current study, a novel niosome based formulation of diallyl disulfide (DADS) was evaluated for its potential to treat disseminated candidiasis in mouse model. Among various non-ionic surfactants tested, niosome formulation prepared using Span 80 was found to be most efficient in the entrapment of DADS. The DADS loaded niosomes had size dimensions in the range of 140 ± 30 nm with zeta potential of −30.67 ± 4.5. Liver/kidney function tests as well as histopathologic studies suggested that noisome-based DADS formulations are safe at the dose investigated. When administered to *Candida albicans* infected animals, the DADS bearing niosomal formulation cleared the fungal burden and increased their survival much efficiently than its free form.

***From the Clinical Editor:*** In this study, a novel niosomal formulation of the antifungal DADS was utilized in a murine candidiasis model, resulting in more efficient fungal clearance compared to the free formulation.


*Key words:* Diallyl disulfide; Niosome; Antifungal efficacy; Candidiasis

*Candida albicans,* in a manner similar to other opportunistic fungal pathogens, causes serious infections, often systemic, among immuno-debilitant subjects such as AIDS patients, people undergoing cancer chemotherapy or those being treated with immune-suppressive drugs (eg., during organ transplantation).[1-4] Incidentally, research and development of novel antifungal drugs did not receive remarkable attention because of the low prevalence of human infection in the pre-AIDS era. The emergence of *Candida* isolates less susceptible to widely used antifungal drugs such as azoles has further aggravated the situation.[5] This necessitates administration of relatively high doses of presently available antifungal drugs, which brings with them a specter of drug-associated toxicity in patients.[6]

Plant-based therapeutics having improved antimicrobial activity with less toxicity, are being increasingly accepted as alternatives to conventional antibiotic therapy.[7] For example, garlic, a traditionally used anti-infective agent, has been widely prescribed in treatment of both fungal and bacterial infections. The wonder herb, garlic, owes its antimicrobial activity primarily to the compound allicin (allyl 2-propene thiosulfinate) formed after the crushing or cutting of garlic cloves.[8-12] As allicin rapidly undergoes degradation that makes its clinical usage difficult, steam-distillation of mashed garlic pulp is carried out to convert allicin to garlic oil (composed of methyl and allyl sulfide derivatives of allicin), which relatively enables its usage in medicinal formulations.[13] Allyl constituents of garlic oil have been evaluated with varying success for their anticancer, antimicrobial, and other pharmacologic activities.[14] Among these, DADS, a member of allyl sulfides family (diallyl sulfide, diallyl disulfide, and diallyl trisulfide)[15-17] has been reported to possess both anti-bacterial as well as anti-fungal activity.[11] An exhaustive study on role of these sulfides suggest that number of

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disulfide bonds might have direct correlation with antimicrobial activity of a given allyl sulfide.[17-19]

The work of Lemar et al.[20] on the antifungal mechanism of DADS against *C. albicans,* as model fungal pathogen, strongly suggests that increased oxidative stress through decreased GSH levels generally mediates the drastic death response induced in the organism. This study further suggests that impairment of mitochondrial respiration elicited by the action of DADS on ATP-synthase and a site(s) located among complexes II-IV in the electron transport chain is also likely to be involved in the killing of *C. albicans*.[20] Despite showing strong antimicrobial activity, hydrophobicity of DADS is considered to be a major hurdle in the development of its suitable dosage form. This is the reason why desirable pharmacologic properties of this wonderful molecule cannot be translated in clinical settings till now.

With a view to overcome the solubility issues of DADS, we developed a non-ionic surfactant based niosome formulation. Niosomes are bilayered structures, made from non-ionic surfactants and can be used as carrier of both hydrophobic as well hydrophilic drugs. They are biodegradable, biocompatible, and more importantly non-immunogenic with minimum toxicity.[21] The niosome mediated delivery has potential to increase efficacy of the associated drug molecules.[22-26] Interestingly, unlike liposomes that are made up of phospholipids, the constituents that are more vulnerable to heat and oxidation mediated degradation, niosomes show better stability with increased shelf life of entrapped drugs.[26,27]

Incidentally, *C. albicans* adapts intracellular parasitism as a strategy to avoid antibody onslaught and resides in macrophages. We envisaged that being particulate in nature the drug bearing niosome would be avidly taken up by macrophages. This eventually will help in targeted delivery of niosome-loaded DADS to pathogen harboring macrophages. To test our hypothesis, a number of non-ionic surfactants of the "SPAN" series (sorbitan monoesters) were screened to prepare desirable DADS-bearing niosome formulations. The ideal formulation was evaluated for favorable entrapment efficiency of the active constituent, as well as other parameters such as acceptable release kinetics, size and zeta-potential. Finally, we examined the potency of DADS niosomal formulation in treatment of experimental systematic candidiasis in Balb/c mice.

## Methods

### *Chemicals*

DADS, cholesterol, sorbitan monoester non-ionic surfactants viz. Span 20 (sorbitan mono laurate), Span 40 (sorbitan mono palmitate), Span 60 (sorbitan mono stearate), Span 80 (sorbitan mono oleate), dicetyl phosphate (DCP), and amphotericin B were purchased from Sigma Aldrich (St. Louis, Missouri). Detection kits for liver and kidney function test parameters were purchased from Span Diagnostics (Gujarat, India). The rest of the chemicals were of analytical grade of purity and procured locally.

### *Strains*

The test strain of *C. albicans* (ATCC 18804) was obtained from ATCC. Yeast-peptone-dextrose (YPD) agar/broth was used for growing the *C. albicans* strain. In addition, for in vitro antifungal activity tests, clinical isolates of *C. albicans* (29 isolates), *C. glabrata* (19 isolates), and *C. krusei* (15 isolates) were obtained from the repository of Jawaharlal Nehru Medical College, Aligarh Muslim University, Aligarh, India.

### *Preparation of DADS-bearing niosomes*

We used sonication method for preparation of Span-based niosomal formulations of DADS after published procedure as standardized in our laboratory.[28] The details of the preparation method have been incorporated in Supplementary Material (available online at http://www.nanojournal.com).

### *Determination of entrapment efficiency of niosome-intercalated DADS*

The entrapment efficiency of DADS was determined by dissolving an aliquot of the in-house prepared niosomal DADS formulation in chloroform:methanol (1:9 v/v) mixture, followed by estimation of DADS content by high performance liquid chromography (HPLC) method employing published procedure.[29] The details of the experimental protocol are given in the Supplementary section.

### *In vitro release kinetics of DADS from niosomal formulation*

Release of DADS from various niosomal formulations was studied using a dialysis method as published earlier.[30,31] Briefly, niosomal DADS formulation (containing 1 mg DADS) was dispersed in 1.0 mL of phosphate buffered saline (PBS) (pH 7.4) and placed in a dialysis bag. It was fully immersed under release medium (PBS, pH 7.4) taken in a glass beaker and kept for gentle stirring on a magnetic stirrer at room temperature (25°C). Aliquots of the medium were withdrawn for analysis of released drug at different time intervals and the volume of the suspension buffer was replenished by adding same volume of fresh medium.

### *Determination of particle size and zeta potential of DADS-bearing niosomes*

The zeta potential gives an indication of the charge acquired by particulate system on its suspension in aqueous medium. In fact, it is a measure of surface charge on a given particle that regulates its half-life both in vitro as well as in vivo. Zeta potential of different formulations was measured by the instrument Zeta sizer Nano ZS (Malvern Instrument Limited, Worcestershire, United Kingdom). Briefly, lyophilized form of the formulation was taken in microfuge tube and the samples were suspended in 20 mM PBS, pH 7.4, and then introduced into the instrument following the guidelines of the manufacturer.

### *Niosome-mediated transfer of entrapped molecules to macrophages*

The successful elimination of intracellular pathogen *C. albicans* requires effective delivery of drug pay load to infected macrophages. We determined uptake of niosome encapsulated fluorescent dye (calcein) by macrophages as a parameter to assess ability of newly developed niosomal formulation to home intercalated drug to *C. albicans* harboring

macrophages. The details of the niosome uptake study have been provided in Supplementary section.

### Animals

For in vivo studies, female Balb/c mice of weight 20 ± 2 g were procured from the institute's animal house facility and were kept on standard pellet diet (Hindustan Lever Ltd.) and water ad libitum. Animals were checked daily for their mortality and morbidity before commencement of the study and only healthy animals were included in the experiments. The techniques used for bleeding, injection as well as for the killing of animals were strictly performed after mandates approved by CPCSEA (Committee for the Purpose of Control and Supervision of Experiments on Animals, Government of India).

### Toxicity assessment of niosomal DADS formulation

Before introducing the novel niosomal formulation of DADS as potential antifungal therapy approach, we determined its intrinsic toxicity issues in both in vitro as well as in vivo system. Preliminarily, acute drug toxicity was assessed through in vitro erythrocyte lysis test, wherein hemoglobin released as a result of membrane leakage or disruption caused by exposure to the drug formulation, was measured.[32] The details of the procedure have been included in the Supplementary Material section.

In vivo hepatic and renal toxicities for free drug as well as niosomal DADS formulations were assessed by administering 10 × multidoses intra-peritoneally (ip) in Balb/C mice (total three doses, at alternate days). Biochemical profiles of serum creatinine, alkaline phosphatase (ALP) and gamma-glutamyl transferase (GGT) were determined using commercial detection kits (Span Diagnostics, India). Blood was collected by retro-orbital puncture from the mice belonging to various experimental groups at day 1 (before administration of first drug dose) and a day after the last dose administration. The serum was separated from blood for determination of creatinine, ALP, and GGT contents according to specific protocols provided by the manufacturer.

The toxic effect of DADS (if there was any) on vital organs (viz, liver and kidney) of the experimental animals was also assessed by histopathologic studies. For histopathologic examination of liver and kidney, mice from each group were fixed by intracardiac perfusion of 10% buffered formalin. Tissue blocks of liver and kidney (1 × 0.2 cm) were processed for paraffin embedding. Tissue sections (10 μm-thick) were stained with regular hematoxylin and eosin stains and examined under light microscope (Olympus-BX 40, Japan) at ×200.

For in vivo toxicity studies the animals were divided in following groups, with eight mice in each group:

Group I No drug (Control)
Group II Free DADS (60 mg/kg bw)
Group III Free DADS (120 mg/kg bw)
Group IV Niosomal DADS (60 mg/kg bw)
Group V Niosomal DADS (120 mg/kg bw)

Where bw stands for body weight of mouse.

### In vitro antifungal testing and minimum inhibitory concentration (MIC) of DADS for Candida spp.

To determine antifungal susceptibility, the *C. albicans* strain used in the study was exposed to amphotericin B (final concentration range of 0.02 to 5 mg/L), a standard antifungal agent using broth microdilution method performed in 96-well round bottom microtitre plates, as per NCCLS recommendations.[32] Further the in vitro antimicrobial activity of DADS against a total of 63 isolates of *Candida spp.* viz *C. albicans* (29 isolates), *C. glabrata* (19 isolates), and *C. krusei* (15 isolates) was determined simultaneously. These isolates were also tested for any resistance to amphotericin B. DADS was solubilized in 5% DMSO and various aliquots of drug solution were further diluted in RPMI 1640 medium (pH 7.0) containing a seeded final inoculum concentration of $2 \times 10^3$ cells/mL. DADS was tested over a final concentration range of 1 mg/L to 100 mg/L. The wells containing fungal inoculum with different concentrations of drugs and appropriate controls (drug-free as well as inoculum-free) were incubated at 37°C for 48 hours. Turbidity was measured spectrophotometrically at 530 nm. The MIC was defined as the lowest concentration of drug that produced 80% reduction in turbidity.

### Preparation of inoculum

For infection purpose, *C. albicans* was cultured in 5% dextrose broth at 37°C for 36 hours. The cell suspension was centrifuged at 5000× g for 15 minutes at 4°C, followed by washing with sterile normal saline.

### Induction of experimental candidiasis

For induction of experimental candidiasis, each mouse was challenged via the tail vein with $1 \times 10^7$ colony forming units (CFU) of *C. albicans* suspended in 200 μL of sterile normal saline (150 mM, pH 7.4). Our pilot studies suggest that this inoculum size consistently produced disseminated infection within 48 hours of injection and ultimately ensuing in death of animals within 7 days postchallenge with infection (data not shown).

### Assessment of antifungal efficacy

For antifungal efficacy, animals were treated with various formulations given by ip route. The efficacy of Span 80 niosomal DADS was assessed by monitoring the survival of the infected animals and determining the residual *C. albicans* burden in their vital organs viz. liver, spleen, and kidney. For survival and residual fungal burden studies two separate but similar experiments were set up simultaneously. In each experiment the infected animals were divided in six different groups as listed below. Each group had 10 animals.

Group I Control (Only PBS)
Group II Control (Sham niosome)
Group III Free DADS (6 mg/kg bw)
Group IV Free DADS (12 mg/kg bw)
Group V Niosomal DADS (6 mg/kg bw)
Group VI Niosomal DADS (12 mg/kg bw)

For survival studies, mortality of the animals was observed twice each day during 50 days of observation. Quantitative

Table 1
Niosome forming ability and %EE of SPAN-based niosomes with regard to DADS

| Surfactant Percent mol | Cholesterol Percent mol | DCP Percent mol | %EE ± SD | | | |
|---|---|---|---|---|---|---|
| | | | Span 20 | Span 40 | Span 60 | Span 80 |
| 100 | 0 | 0 | 9.2 ± 2.4 | No vesicles | 12.3 ± 4.7 | No vesicles |
| 70 | 30 | 0 | 25.3 ± 3.8 | 27.9 ± 2.7 | 49.5 ± 4.2 | 23.4 ±.4.2 |
| 50 | 50 | 0 | 36.52 ± 4.6 | 35.3 ± 3.4 | 65.7 ± 2.6 | 72.7 ± 4.2 |
| 60 | 30 | 10 | 17.8 ± 3.9 | 32.4 ± 4.3 | 37.2 ± 4.3 | 29.3 ± 4.3 |
| 50 | 40 | 10 | 15.2 ± 5.15 | 34.2 ± 3.3 | 51.8 ± 4.5 | 32.5 ± 4.7 |
| 47.5 | 47.5 | 5 | 35.2 ± 4.17 | 41.2 ± 2.8 | 68.6 ± 3.3 | 74.5 ± 3.2 |

DADS, diallyl disulfide.

assessment of the fungal burden in various vital organs viz. liver, spleen, and kidney was performed following published procedure.[33] Antifungal treatment was commenced 24 hours postchallenge of animals with *C. albicans* infection. Treatment was repeated at day 3 and day 5 postinfection. The animals (three from each group) were killed on day 7, 12, and 15 postinfection, and vital organs viz. liver, spleen, and kidney were taken out aseptically. The organs were washed extensively with hypotonic buffer, homogenized, and serially diluted with normal saline. Various dilutions of each organ homogenate were dispersed on YPD agar plates containing gentamycin to avoid bacterial contamination. After incubation of 12-24 hours at 37°C, the colonies were counted and the fungal load was determined by multiplying with the dilution factor.

### *Tissue distribution studies*

Tissue distribution of DADS in various organs was determined following published procedure.[34,35] Both normal as well as infected mice were administered with free as well as niosomal DADS (12 mg/kg) via intravenous route. The details of the procedure are available in Supplementary Material section.

We also determined plasma level of DADS on administration of DADS bearing niosome in the experimental animals. The animals were injected with 10× amount of therapeutic dose via intraperitoneal route in both normal healthy as well as *C. albicans* infected mice. Blood was withdrawn by retro orbital puncture at different time intervals. Serum was separated by centrifugation at 795× g for 5 minutes. It was then precipitated with methanol and centrifuged at 8832× g for 10 minutes. Supernatant was taken out and analyzed for DADS content by HPLC method.

### *Statistical analysis*

The results were analyzed with one-way analysis of variance (ANOVA) of mean values with the Student *t* test. *P* value of $< 0.05$ was considered statistically significant.

## Results

### *Effect of composition on the formation and entrapment efficiency of DADS-containing niosome formulations*

The DADS-loaded niosomes were prepared using various molar ratios of the Span surfactants, cholesterol, and DCP. The percent entrapment efficiency (% EE) for DADS is summarized in Table 1. Niosomes prepared using Span 20 and Span 40 had very low % EE irrespective of the molar ratios of constituents used. The maximum entrapment was achieved by Span 80-based niosomes (74.5 ± 3.2%) at molar ratio of 47.5:47.5:5::SPAN 80:cholesterol:DCP and 72.7 ± 4.2% at 50:50:0::SPAN 80:cholesterol:DCP % molar ratios, respectively. This was followed by Span 60 niosomes (68.6 ± 3.3% and 65.7 ± 2.6% for molar ratios 50:50:0 and 47.5:47.5:5::SPAN 60:cholesterol:DCP, respectively). Exclusion of DCP during preparation of either Span 60 or Span 80-based niosomes resulted in formation of large aggregates within 24 hours of preparation. Based on earlier reports that Span 80 and Tween 80 niosomes are more stable than other detergents and our supporting data of entrapment efficiency, the Span 80-based niosomes (47.5:47.5:5 molar composition) were subsequently chosen for characterizations and assessment of efficacy studies against experimental murine candidiasis.

### *Particle size, zeta potential and shape of DADS-bearing Span 80 niosomes*

The zeta-potential of DADS-loaded Span 80 niosomes was found to be −30.67 ± 0.45 mV. The size of the novel DADS-bearing niosome formulation was established using zeta average diameter analysis. It shows niosome to possess size range of 140 ± 30 nm.

### *Niosome-mediated transfer of entrapped molecules to macrophages*

To know fate of calcein loaded niosomes after their uptake, they were incubated with mouse J774 A.1 cells and subsequently examined by fluorescence and phase contrast microscopic techniques. As evident from Figure 1, *A* and *B*, the incubation of niosomes with macrophages resulted in punctate fluorescence suggesting their uptake by active endocytosis of the target cells. Both normal and infected macrophages were able to internalize calcein-loaded noisome; however, uptake was more obvious in case of infected macrophages.

### *Release profile of niosomal DADS*

Figure 2 shows the release pattern of DADS from Span 80 niosomes wherein approximately 20% of the intercalated drug was released in 24 hours and around total 33% of the drug was released in 72 hours. The release profile suggests that noisome-based formulation releases the drug in a sustained manner.

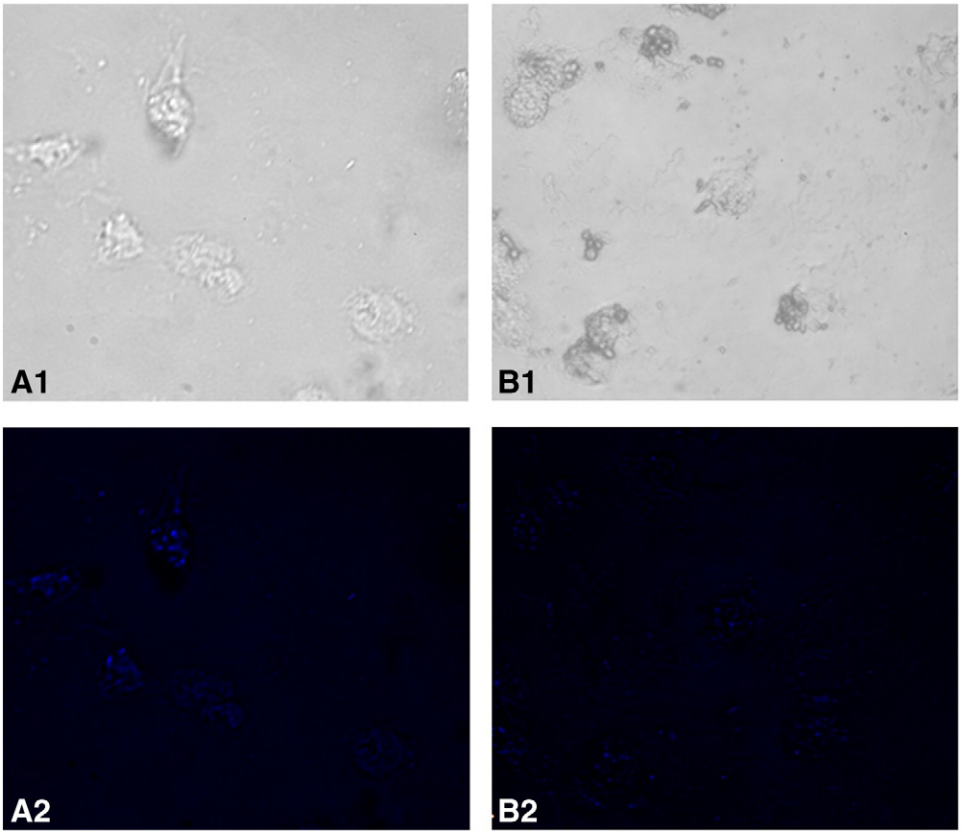


Figure 1. Fluorescence light micrographs of the macrophages J774 A.1 after their interaction with calcein (fluorescent probe) loaded niosome. Fluorescent and Phase contrast light micrographs of normal macrophages **(A1 & A2)** and *C. albicans* **(B1 & B2)** infected macrophages interacted with calcein loaded niosome for 60 min at 37°C as described in *Methods* section.

*Toxicity tests*

(a) *In vitro hemolysis test.* The possibility of Span 80 niosomal DADS mediated lysis of erythrocytes was assessed using a dose that was almost 10 times greater to that of the therapeutic dose used in chemotherapy of the infected animals. Figure 3 displays extent of erythrocyte lysis induced by free as well as niosomal DADS with appropriate controls (Triton X-100, empty sham niosomes and DMSO). As niosome releases entrapped DADS molecules in regulated manner, it does not cause any lysis of RBCs, whereas free form of DADS had induced significant lysis.

(b) *In vivo toxicity of DADS formulations.* Renal and hepatic toxicities of the in-house developed formulations were measured after the administration of a 10 times higher concentration of DADS when compared with that of the therapeutic dose used in in vivo efficacy experiments. As evident from the data, there was no apparent change in the RFT and LFT parameters of the animals receiving the DADS formulation when compared with the control (Table 2).

Histopathologic studies revealed that on treatment with Nio-DADS at 12 mg/kg dose, the liver appeared to possess normal hepatic lobular architecture and hepatocyte laminae (Figure 4, *B*). Sinusoids were mildly dilated because of acute hyperaemia. Hepatocytes showed smooth contour and uniform staining without any sign of degeneration or necrosis and were identical with the healthy control animal, except for the dilated sinusoids (Figure 4, *A* and *B*).

Similarly, renal parenchyma of the kidney from drug treated animals (Figure 4, *D*) was quite comparable with the healthy animals (Figure 4, *C*). The kidney showed normal renal corpuscles in terms of glomerular mass, its cellularity, and urinary space as well as renal tubules where proximal convoluted tubule (PCT) and distal convoluted tubules (DCT) could be identified with their intact characteristic lining cells as well as interstitial space.

*In vitro antifungal testing and MIC of DADS*

The MIC of amphotericin B against *C. albicans* strain (ATCC 18804) was 0.125 μg/mL, which suggests that the strain is antifungal susceptible. Similarly all the 63 *Candida* isolates consistently showed amphotericin B MIC in the range of 0.15-3.68 μg/mL indicating absence of any resistance to standard antifungal drug (data not shown). DADS showed MIC of 6 ± 0.8 mg/L for the *C. albicans* strain (ATCC 18804). For the 63 clinical isolates of *Candida spp.* the DADS MIC was 7 ± 1.7 mg/L for *C. albicans* isolates, 10 ± 2.54 mg/L for the

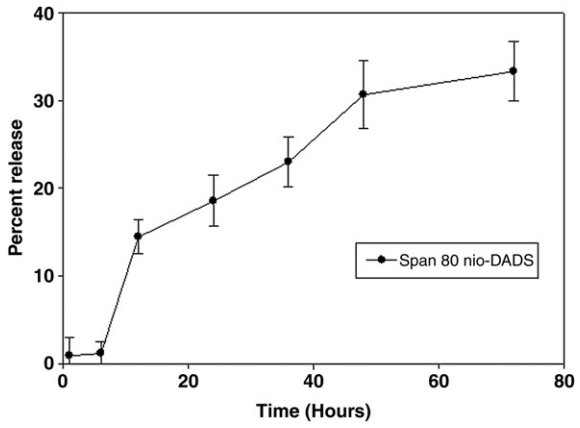


Figure 2. Release of DADS from DADS-based niosomes. Niosomal DADS suspension was incubated in sterile phosphate buffer saline (10 mM phosphate, 150 mM saline, pH 7.4). The amount of DADS released at different time points was spectrophotometrically analyzed at 254 nm as described earlier in *Methods* section. A standard curve of the drug was plotted at 254 nm by determining the area under curve corresponding to the known increasing amount (10-500 ng) of the drug. Release runs were continued for 72 h. The absorbance of the collected samples was measured at 254 nm. The calculated amount of the released drug was plotted against time. Data are means ± SD of five independent experimental values.

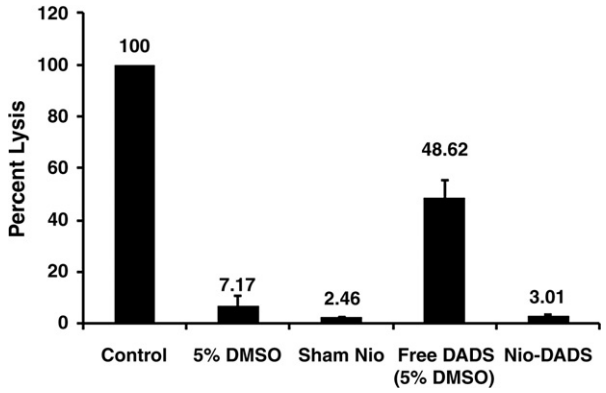


Figure 3. Erythrocyte lysis test for DADS-bearing niosomes. The extent of damage incurred to blood erythrocytes by DADS formulations was measured as percentage lysis of total erythrocytes used in the individual sample.

*C. glabrata* isolates, and 11.8 ± 3.62 mg/L for the *C. krusei* isolates (Supplementary Table S1).

### *Assessment of fungal load in vital organs of infected animals after treatment with niosomal DADS formulation*

The severity of *C. albicans* infection was assessed by culturing the liver, spleen, and kidney homogenates, of animals belonging to various experimental groups onto YPD agar plates. As shown in Figure 5, *A*, there was significant decrease in the fungal load in liver, spleen, and kidney respectively from animals of Group VI treated with Span 80 niosomal DADS (12 mg/kg bw) when compared with animals of Group IV treated with 12 mg/kg bw free DADS treatment ($P < 0.04$). Untreated control animals of Group I did not survive beyond 5 days and were not available for killing for determining fungal load.

Table 2
Concentrations of creatinine, alkaline phosphatase (ALP), and gamma glutamyl transferase (GGT) in plasma of mice after drug therapy

| Groups | Percent Creatinine ± SD | ALP (in KA Unit) | GGT (IU/L serum) |
|---|---|---|---|
| Control | 0.58 ± 0.09 | 0.00[a] | 1108.5 ± 113.84 |
| Free DADS (120 mg/kg) | 1.04 ± 0.17 | 3.78 ± 0.059 | 1265.0 ± 50.91 |
| Niosomal DADS (120 mg/kg) | 0.81 ± 0.29 | 0.51 ± 0.09 | 1064.05 ± 80.53 |

DADS, diallyl disulfide; SD, standard deviation.
[a] Amount of ALP is calculated by subtracting the value of control from test. Hence, it has been shown as 0.

### *Survival studies*

As shown in Figure 5, ***B*,** the animals treated with niosomal formulation of DADS (Nio-DADS) at dose of 12 mg/kg bw (Group VI) showed a survival rate of 80% as noted up to day 50 postinfection, whereas 50% animals survived in Group V (animals that were treated with niosomal-DADS at dose of 6 mg/kg bw). None of the animals survived beyond the15th day postinfection in the Group IV that were treated with free form of DADS at dose of 12 mg/kg bw. The Nio-DADS formulation (12 mg/kg bw as well as 6 mg/kg bw) was more effective when compared with free DADS in improving survival rate of treated animals. The animals belonging to control groups (sham niosome and PBS treated) succumbed to death within 7-8 days postchallenge with infection.

### *Tissue distribution of DADS in vital organs*

Figure 6, *A*, shows that on administration of free DADS, its concentration was increased in liver and spleen 1 hour postinjection but decreased significantly after 24 hours, whereas the drug concentration in kidney and lungs remained lower in this period. The DADS concentration in the animals receiving niosomized form was less in liver and spleen when compared with the free form of drug at 1 hour but increased by 24 hours postinjection indicating steady and gradual accumulation of drug to both organs. Figure 6, *B*, shows that DADS concentration was higher in all the organs when it was administered to the *C. albicans* infected animals. To determine serum concentration of DADS, animals were injected with free or niosomal DADS at the dose of 12 mg/kg bw. Although statistically nonsignificant, administration of free-DADS resulted in higher serum concentration, whereas niosomal DADS treated group had relatively low concentration at various time points (Figure 6, *C*). A time dependant increase in DADS concentration in the serum was observed in both the groups. On the other hand, increased plasma half-life of DADS was more noticeable in the case of the niosomal drug treatment of the infected animals when compared with the free drug indicating that DADS distribution in bio-phase is remarkably influenced by its incorporation in niosomes. Overall the significant altered distribution of the entrapped drug

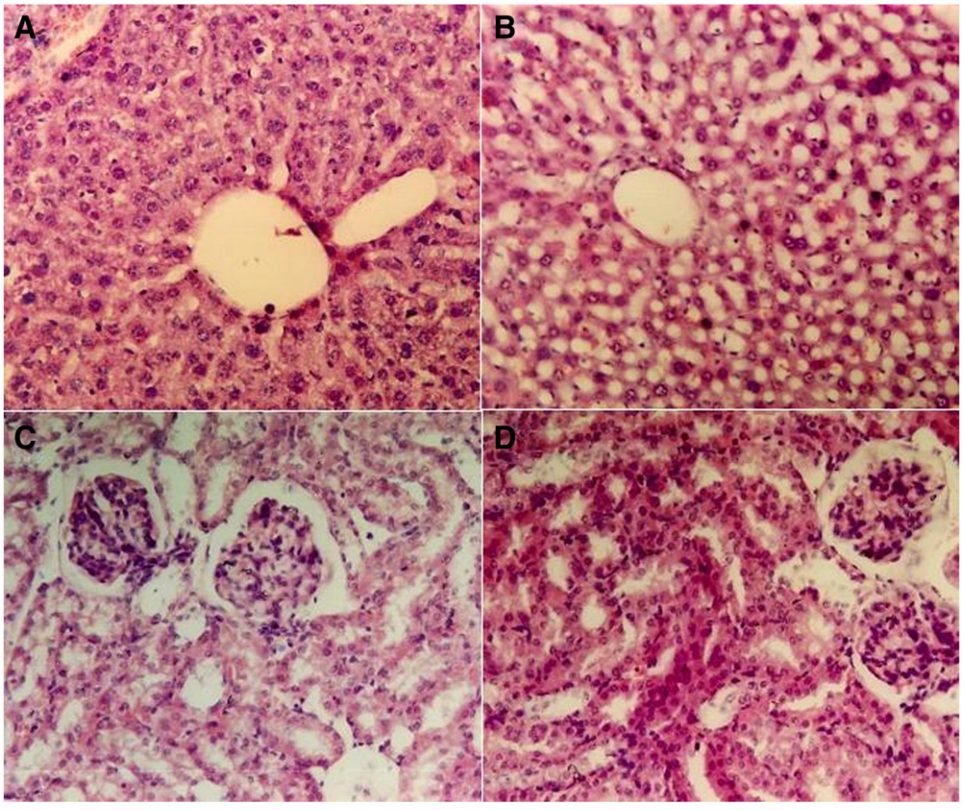


Figure 4. Histopathologic studies. Photomicrographs of mice liver samples from control **(A)** and niosomal DADS (12 mg/kg bw) treated group **(B)**. In the treated group the lobular architecture, hepatic laminae and contour of hepatocytes are intact with usual presence of red blood cells in the sinusoids. There is no sign of hepatocyte necrosis. Photomicrographs of kidney samples from control **(C)** and niosomal DADS (12 mg/kg bw) treated group **(D).** Treated group shows intact renal corpuscle with normal glomerular cellularity, urinary space, and lining cells of renal tubules. ×200, Stain H & E.

in healthy versus *C. albicans* infected animals suggest that the infection with *C. albicans* clearly influence the distribution of the drug in various organs of the host animal (Figure 6, *C*).

## Discussion

In the current study, we have tried to establish antifungal potential of DADS, a plant based compound against systemic murine candidiasis in model animals. Keeping into consideration the fact that poor solubility of DADS hampers development of suitable dosage form that can otherwise facilitate its systemic distribution upon administration in the host, it was envisaged that incorporating DADS in the bilayer of niosomes, which are particulate vesicular entities can enable solubilization of the hydrophobic compound and modify its systemic distribution in the body.

To confirm our hypothesis, we developed niosome-based DADS formulation and evaluated its antifungal efficacy against experimentally induced disseminated *Candida* infection in mice. Span 80-based DADS niosomal formulation showed favorable drug entrapment efficiency, desirable surface charge, particle size and drug release kinetics (Figure 2). Recently it has been reported that higher fatty acid can also inhibit a range of microbes including *C. albicans*.[36,37] The unsaturated fatty acids are found to be more potent antifungal agents than their saturated counterpart, the reason why we opted for Span 80, an oleic acid ester of sorbitan over Span 60, which is an ester of stearic acid in spite of the fact that both Span 60 as well as Span 80 had comparable drug entrapment efficiency.[38] DCP was used to impart negative zeta potential to the prepared niosomes that may help in preventing or delaying aggregation when particles are suspended in aqueous medium. Besides, negative zeta potential may also increase plasma half-life of the niosome-based formulation. The surface properties and sustained drug kinetics from niosome-based formulation are desirable features that can have direct effect on efficacy of the drug.

To establish that in-house prepared niosomal formulation can deliver its contents to macrophages, the calcein (a water soluble fluorescent probe) bearing niosomes were incubated with macrophages in vitro. The interaction eventually resulted in punctuate fluorescence pattern referring to co-localization of niosome bound calcein in endo-lysosomal compartment of the macrophages, the site that *C. albicans* (yeast form) used as dwelling shelter inside the host (Figure 1, *A* and *B*).

Before proceeding for in vivo efficacy studies, we tested the niosomal DADS formulation for inherent toxicity issue if there was any. In this regard, toxicity of the formulation to living cells as revealed by in vitro erythrocyte lysis test (Figure 3) suggests that niosomal DADS induces negligible damage to red blood cells even at 10× therapeutic dosage. Further, results of in vivo liver and renal function tests (Table 2) confirm that niosomal

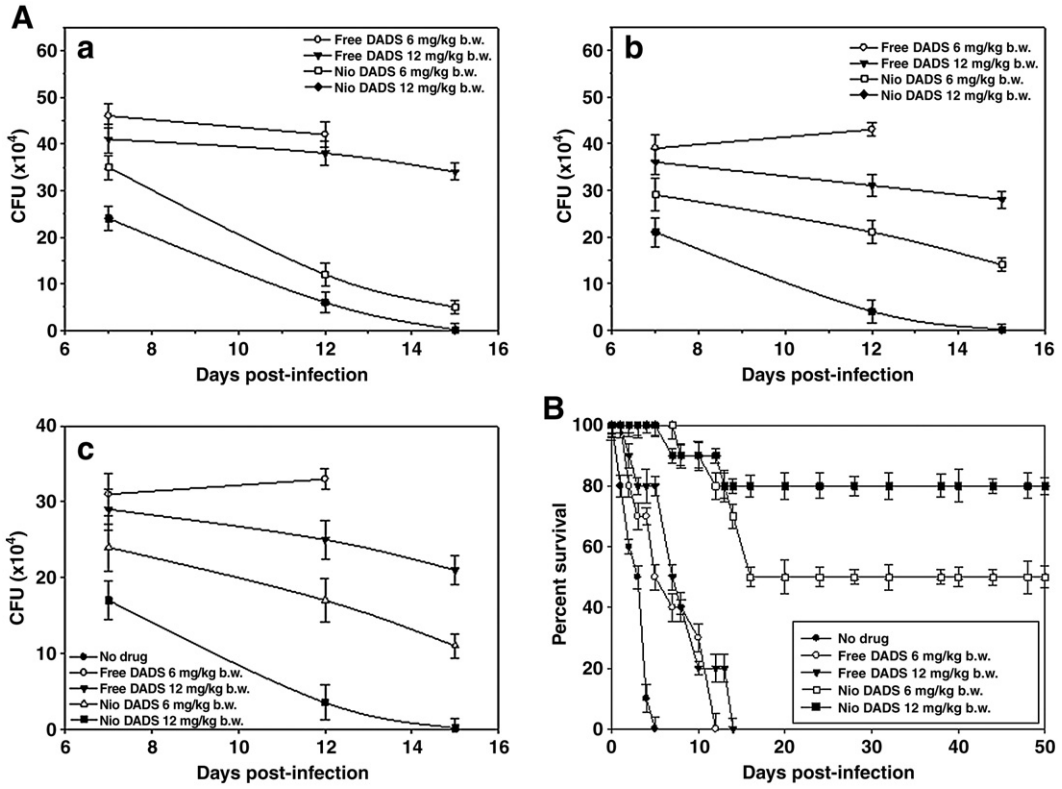


Figure 5. **(A)** Span 80 niosomal DADS mediated fungal load reduction in vital organs of *C. albicans* infected mice. Animals were challenged with $10^7$ CFU of *C. albicans* and subsequently treated with various forms of DADS as described in *Methods*. Antifungal efficacy was determined as residual fungal load in the respective vital organ plotted against number of days postinfection. No animal survived in untreated control (No drug) beyond 5th day postinfection, therefore none was available for sacrifice. Fungal burden in **liver (a)** Nio-DADS (12 mg/kg bw) versus Free DADS (12 mg/kg bw), $P < 0.03$, **spleen (b)** Nio-DADS (12 mg/kg bw) versus Free DADS (12 mg/kg bw), $P < 0.03$, and **kidney (c)** of niosomal DADS treated mice Nio-DADS (12 mg/kg bw) versus Free DADS (12 mg/kg bw), $P < 0.05$. **(B)** Increase in survival of animals by Span 80 niosomal DADS. Animals were infected with $10^7$ CFU and subsequently treated with various forms of DADS as described in *Methods*. The data is the mean of five sets of different experiments ± SD. Nio-DADS (12 mg/kg) versus Free DADS (12 mg/kg), $P < 0.001$, Nio-DADS (6 mg/kg) versus Free DADS (6 mg/kg), $P < 0.001$.

DADS imparts mild toxicity to host liver and kidney when compared with its free form. In fact, non-ionic surfactant Span 80 used in preparation of niosome is a Food and Drug Administration (FDA)-approved food additive thus rules out any intrinsic toxicity constraint. The possible toxicity issues related with formulation were further excluded by histopathologic studies in which features of liver and kidney from DADS-treated animal (12 mg/kg bw) appeared very much similar to that of control healthy animal.

Our pilot studies suggested that in a manner similar to liposome-based formulation, DADS being hydrophobic in nature gets readily incorporated in the niosome bilayer. We established preferential distribution of DADS in niosome bilayer by sonication-resonication method that accounts for the presence of more than 95% of DADS in bilayer rather than aqueous core compartment (data not shown).

The efficacy studies suggest that, the Span 80-based formulation was dramatically effective in clearing fungal burden in liver, spleen and kidney of infected animals (Figure 5, *A*). In comparison, same dose of free form of DADS failed to eliminate fungal load in various vital organs of the experimental animals (Group III and Group IV). The in vivo survival study (Figure 5, *B*) showed a significant reduction of mortality in animals treated with niosomal DADS. The higher efficacy of inhouse prepared DADS formulation was found to have direct correlation with specific distribution of the niosome intercalated DADS to infected organs mainly (Figure 1).

As observed in the tissue and plasma distribution studies (Figure 6), DADS accumulation in the vital organs and blood is influenced by its niosomization as well as presence of infection in the host animals. Incorporation of DADS in niosomes ensured its accretion in macrophage-rich organs that was more pronounced when the animals were infected with *C. albicans*. This is mostly because of the fact that subsequent to establishment of infection, body immune system recruits, and signals various immune cells to accumulate at the site of infection.[39,40] The improved efficacy of niosome encapsulated DADS can also be correlated to the uptake of DADS-loaded particulate niosomes by the pathogen-harboring macrophages, whereas free form of DADS probably has inadequate access to macrophages and hence find it difficult to reach to the infection sites. In fact, macrophages seem to act as secondary depot for niosomal DADS and facilitate its distribution to the pathogen harboring organs of the host.

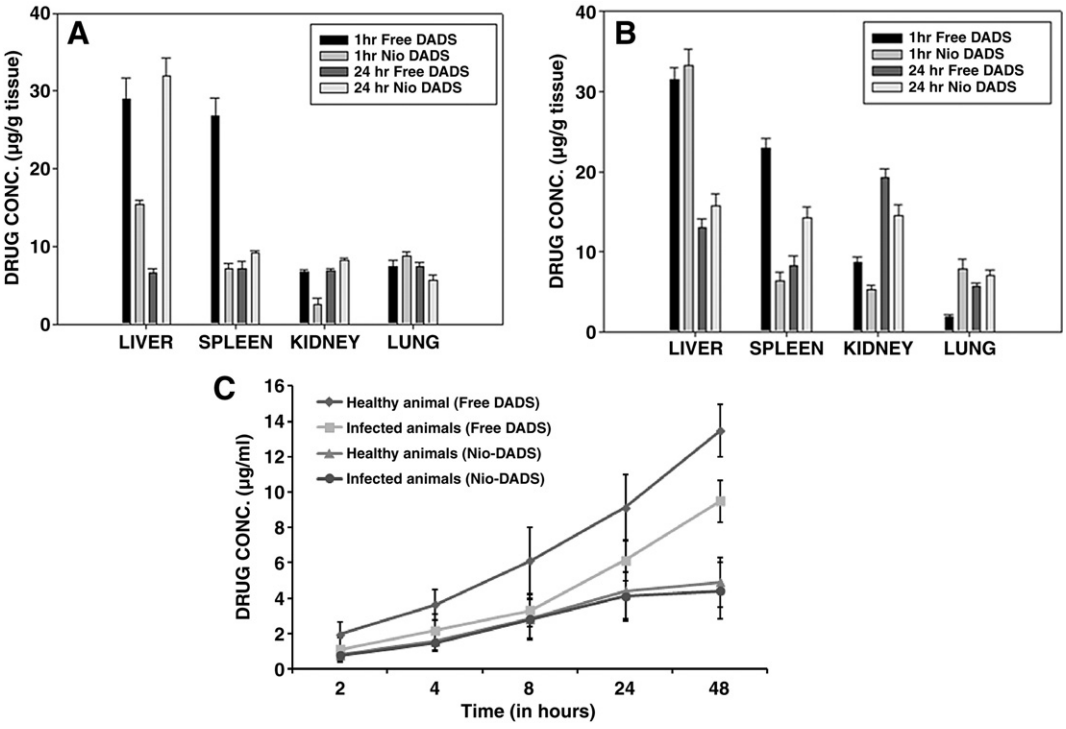


Figure 6. Tissue distribution study. DADS tissue distribution in liver, spleen, kidney, and lungs at 1 and 24 h posttreatment in un-infected **(A)** and infected **(B)** animals. DADS plasma concentration: DADS in free and niosomised form, was administered by ip route in healthy and infected animals and blood was withdrawn at different time intervals. Plasma DADS concentration in healthy and infected animals **(C).** Drug concentration in the organs and plasma was analyzed by HPLC as described in *Methods* section.

Recently, we have demonstrated efficacy of novel formulation of DAS,[41,42] another member of allyl sulfide based essential oil present in garlic extract. However, with the emergence of azole resistant isolates, one cannot rule out possibility that MDR-ABC transporters[43] present in *C. albicans*; can extrude a variety of structurally unrelated compounds including DAS. The drug efflux will not allow attainment of effective concentration of DAS inside the cell. In the current study, we selected a far more potent analog of this family viz, DADS with the assumption that if retained inside the cell even at low concentration its presence may lead to successful elimination of pathogen from systemic circulation. In fact, earlier reports have shown that entrapment of drug in lipid-based delivery systems not only effectively targets multidrug-resistant cells but also remains unaffected by P-glycoprotein-mediated drug efflux.[44]

The data of the current study offers an efficacious, negligibly toxic, and cost-effective alternative to the presently used allopathic drug formulations that have undesirable side effects when administered at pharmacologically effective dosage regimens. The promising findings of the current study corroborate our approach, whereby therapeutic efficacy of DADS can be enhanced by incorporation into niosomes. We hence infer that niosomal formulation of DADS possesses extra advantages over free form of DADS for the treatment of experimental candidiasis because of reduced toxicity and ability to facilitate drug accumulation at RES sites as well as prolonged and sustained release of the DADS over extended time period. Further, niosomal form of DADS are ought to offer great promise in treatment of fungal infections in immuno-compromised persons who usually need prolonged administration of antimicrobial agents when compared to the healthy subjects.

More detailed study and optimizations to elucidate the mechanism of action of DADS, incorporation of target specific ligands (antibody, aptamer) on the surface of the DADS-loaded niosomes to enable active targeting to the site of infection thereby achieving better efficacy, and further amendments to increase shelf life of the formulation are needed to enable development of an effective nature-derived antifungal formulation that can provide an alternative to the presently available antifungal drugs.

## Acknowledgment

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## Appendix A. Suplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.nano.2012.07.004.

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