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Design, development and synthesis of mixed bioconjugates of piperic acid–glycine, curcumin–glycine/alanine and curcumin–glycine–piperic acid and their antibacterial and antifungal properties

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Abstract—In the present communication different curcumin bioconjugates viz. 4,4′-di-*O*-glycinoyl-curcumin, 4,4′-di-*O*-D-alaninoyl-curcumin, 4,4′-di-*O*-(glycinoyl-di-*N*-piperoyl)-curcumin, 4,4′-di-*O*-piperoyl curcumin, curcumin-4,4′-di-*O*-β-D-glucopyranoside, 4,4′-di-*O*-acetyl-curcumin along with piperoyl glycine, have been synthesised and characterised by spectra UV, ^{1}H NMR and elemental analysis. All the covalent bonds used are biodegradable. This makes these derivatives as potent prodrugs, which can get hydrolysed at the target sites. These bioconjugates were tested in vitro against different bacteria and fungi. The 4,4′-di-*O*-(glycinoyl-di-*N*-piperoyl)-curcumin and 4,4′-di-*O*-acetyl-curcumin are more effective than *Cefepime*, an antibacterial drug available in market, at the same concentration. The 4,4′-di-*O*-(glycinoyl-di-*N*-piperoyl)-curcumin and 4,4′-di-*O*-piperoyl curcumin had antifungal activity in vitro almost comparable with *fluconazole*, the most popular antifungal drug. The enhanced activity of these bioconjugates vis-à-vis the parent molecule that is curcumin may be due to improved cellular uptake or reduced metabolism of these bioconjugates resulting in building up of enough concentration inside the infected cells. It opens a new era for exploring suitably designed curcumin bioconjugates as potential antibacterial/antifungal drugs.

1. Introduction

Turmeric (*Curcuma longa*), a common Indian dietary pigment and spice has been shown to posses a wide range of therapeutic utilities in the traditional Indian medicine for external/internal wounds, liver diseases (particularly jaundice), blood purification, inflamed joints (rheumatoid arthritis).[1–5] Curcumin (1,7-bis (4-hydroxy-3-methoxyphenyl)-1,6-heptadiene-3,5-dione/diferuloyl methane) the main pigment of turmeric and its other derivatives have been reported to have antioxidant and various biological activities. It has been reported to have antioxidant properties.[6–8] In our previous publications[9,10] we have highlighted the medicinal importance of curcumin. Turmeric and curcumin have been shown to inhibit carcinogen-induced mutations in the Ames assay and the formation of tumour in several experimental systems.[11–16] As a known antioxidant, curcumin exhibits antiproliferative capability and thus is identified as a potent tool in cancer therapy.[17–20] Curcumin has also been reported to decrease total cholesterol and *LDL* cholesterol level in serum and to increase the beneficial HDL cholesterol level.[21] It has been claimed that curcumin inhibits HIV-I integrase protein, thus potentially preventing HIV-I from infecting CD-4 and CD-8 cells.[22] The other attractive features of curcumin to explore, as a vulnerary agent is that despite being eaten daily for centuries in Asian countries, curcumin has not been reported to be toxic.[2]

Piperine (1-piperoyl piperidine) an alkaloid present in *Piper nigrum-L* (black pepper) has for long been used widely in Auerveda, an Indian system of medicine.[23] Piperine shows important pharmacological activity viz. antiasthmatic property along with vasak leaves *Adhotoda vasica*,[24] antifertility,[25] CNS depressant and anti-inflammatory[26–28] activities and inhibition of hepatic monooxygenase and UDP–glucoronyl transeferase.[29,30] In addition, piperine has also been shown to enhance the bioavailability of drugs like spateine, curcumin,

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barbiturates and oxyphenyl butazone, zoxazolamine, propanlol and theophylline in animal experiments.[21,23,27,30–35]

The traditional knowledge that piperine when mixed with curcumin enhances the bioavailability of latter several times led us to link the two covalently.[21,23] For such a linking there were two alternatives, (i) to link piperic acid obtained by hydrolysis of piperine[36] to the two free phenolics of curcumin via ester links or (ii) to link piperic acid to the diglycinoyl curcumin reported earlier from this laboratory[9,10] via two amide links.

It is well known that curcumin has very poor bioavailability,[21] cellular uptake is slow and it gets metabolised fast once inside the cell, requiring repetitive oral doses in order to achieve significant concentration inside the cells for therapeutic activity. Moreover, the transportation across the cellular membrane of the infected cells is also changed, which necessitates the molecule to be transported to be well internalised with the changed environment of the cell. The past decade has seen extensive use of new polymers, cyclodextrins, liposomes and lipid-based drugs and viral vectors for targeted delivery of drugs.[37–43]

One of the easily accessible approaches was to make bioconjugates of curcumin by covalent attachment with such ligands, which internalise with cellular environment of infected cells. The amino acids, for example, glycine and D-alanine are essential components of bacterial cells. Glucose and acetic acid being food components are also well recognised. These components when linked covalently to curcumin molecule may act as carrier molecules as the cells recognise these molecules and thereby might increase the intracellular delivery of curcumin. The conjugates also are likely to be metabolised comparatively slowly. Therefore our objective was to synthesise curcumin bioconjugates with amino acids, piperic acid, glucose and acetic acid. These curcumin bioconjugates can serve dual purpose of systemic delivery as well as act as therapeutic agents against infectious diseases. The biodegradable bonds used here in (ester amide and glucoside) are likely to get hydrolysed in the living system releasing curcumin at the diseased site.

2. Results and discussion

Curcumin molecule has three functional groups viz. two phenolic and one active methylene group, which make excellent sites for any chemical modification including covalent attachment of biomolecules for synthesising bioconjugates. In the present work only the phenolic groups have been used since the C–C bonds with active methylene group is not biodegradable. The ligands selected were glycine, D-alanine, glucose, acetic acid and piperic acid. The latter was obtained from the hydrolysis of the alkaloid piperine. The main objective for preparing these bioconjugates was:

(i) To ease the transmembrane passage of curcumin, in order to build its significant intracellular concentration.
(ii) To decrease the rate of metabolism of curcumin inside the cell.
(iii) To enhance the hydrophilicity of the molecule.
(iv) To have a biodegradable linkage in the bioconjugate, which could be degraded by the cellular enzymes and curcumin released at the drug target site (pro-drug).

In order to achieve these objectives curcumin was covalently linked to amino acids viz. glycine and D-alanine. Although both are essential amino acids the latter is specific component of many bacteria and therefore must be recognised by bacterial cells. The two conjugates, one with each amino acid, that is, glycine and D-alanine were prepared involving the two free phenolic groups of curcumin, these ester linkage are biodegradable, giving prodrug character to the conjugates and also enhance their hydrophilicity.

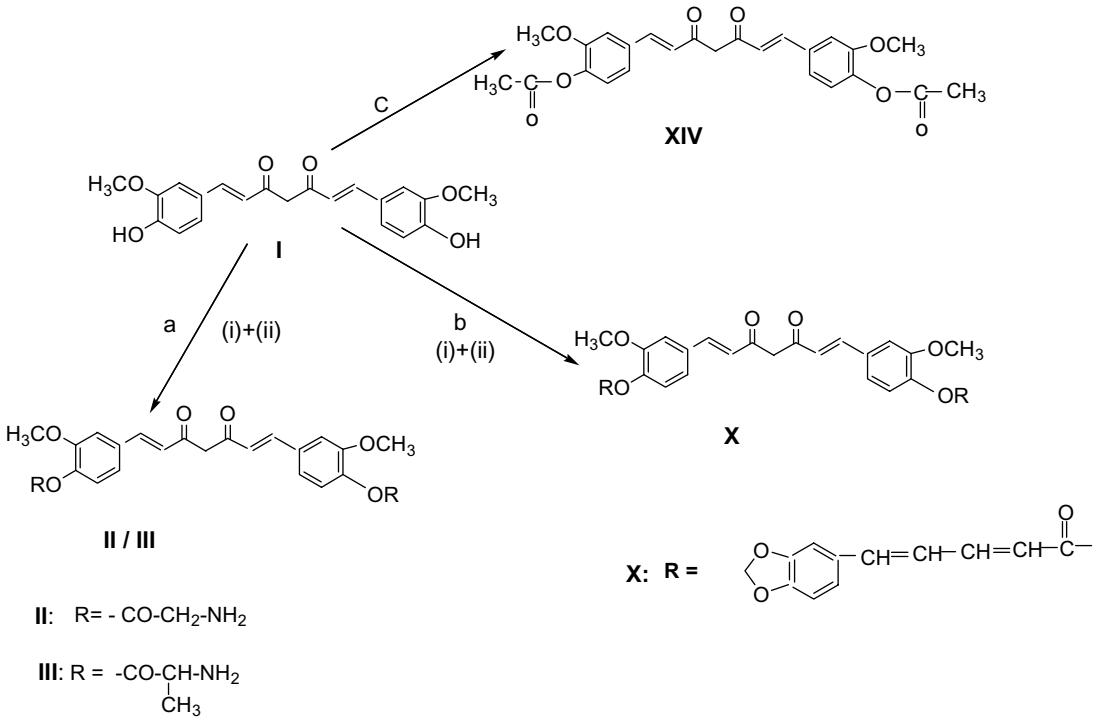
During the synthesis the amino functions of glycine and alanine were protected with phthalic anhydride as *N*-phthaloyl glycine and *N*-phthaloyl alanine and the carboxy groups, were activated by treating with thionyl chloride to get corresponding acid chlorides. The reaction with curcumin in 2:1 molar proportion in pyridine gave the desired products (Scheme 1).

One of the main drawbacks of these synthesis was the relatively poor yields of the final bioconjugates. In order to improve the yields we have developed an alternative approach for these syntheses. The syntheses were carried out in aqueous medium, that is, aqueous phase synthesis of curcumin amino acid-bioconjugates especially of (**II**) and (**III**) gave excellent results (yield ~90%).

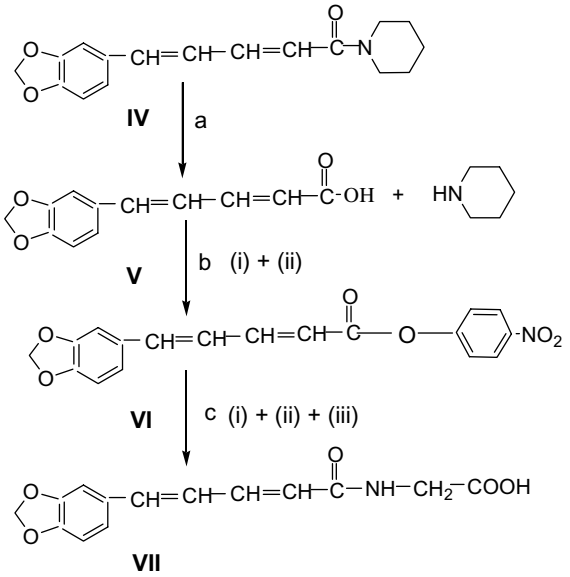
Piperic acid (**V**) was obtained by alkaline hydrolysis of piperine (**IV**), the alkaloid present in black pepper.[36] The –COOH function of piperic acid (**V**) was esterified (activated) by reaction with *p*-nitro phenol by addition of dicyclohexylcarbodiimide (DCC) in presence of pyridine and triethyl amine to make the solution basic to get the corresponding activated ester (**VI**). The activated piperic acid was then reacted with glycine and 4,4′-di-*O*-glycinoyl curcumin in 1:1 and 1:2 molar proportions, respectively, using DCC/DMAP to yield piperoyl glycine (**VII**) and 4,4′-di-*O*-[*N*-piperoyl–glycinoyl]-curcumin (**VIII**) (Schemes 2 and 3). This approach that is to use the *p*-nitro phenyl ester of acid in place of the corresponding acid chloride or anhydride to get the amide bond (–CO–NH) is well established strategy in peptide synthesis. The completion of reaction could be assessed by precipitation of DHU (dicyclohexylurea).

Curcumin was also directly attached with piperic acid using piperoyl chloride in 1:2 molar ratio to get the 4,4′-di-*O*-piperoyl curcumin (**X**).

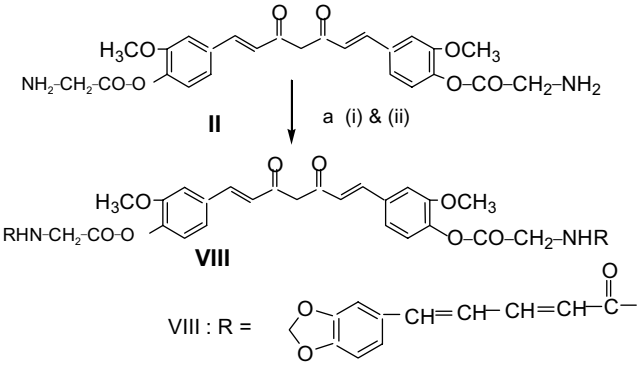
To synthesise 4,4′-di-*O*-(β-D-glucopyrano)-curcumin (**XIII**), penta-*O*-acetyl-D-glucopyranose (**XI**) was prepared and taken in dry dichloromethane and saturated with hydrogen chloride gas with stirring. The solvent was removed at reduced pressure to obtain the 1-α-chloro-2,3,4,6-tetra-*O*-acetyl-glucopyranose (**XII**). Cur-



Scheme 1. Synthesis of di-*O*-glycinoyl curcumin (**II**); di-*O*-alaninoyl curcumin (**III**); (a) i. 10% NaOH/*N*-phthaloyl glycinoyl chloride/*N*-phthaloyl alaninoyl chloride/6 h/0 °C; ii. NH_3–pyridine (9:1) v/v; 1 min. Synthesis of 4,4′-di-*O*-piperoyl-curcumin (**X**): (b) i. pyridine/piperoyl chloride (**IX**) rt/6 h. Synthesis of di-acetate derivative (**XIV**) of curcumin: (c) 10% NaOH/crushed ice/acetic anhydride.



Scheme 2. Synthesis of piperic acid (**V**); *p*-Nitro phenyl ester of piperic acid (**VI**) and piperoyl glycine (**VII**): (a) Ethanolic KOH (2 N); 2 h reflux/HCl; (b) i. *p*-nitrophenol/pyridine/TEA ii. DCC; (c) i. Glycine/pyridine/(**VI**), ii. DCC/DMAP.

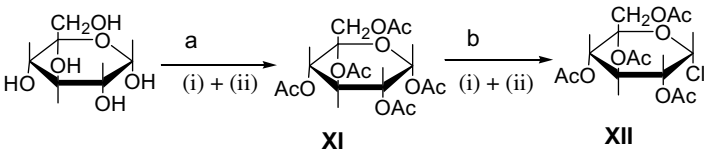


Scheme 3. Synthesis of 4,4′-di-*O*-[glycinoyl-di-*N*-piperoyl]-curcumin (**VIII**) (a) i. *p*-nitro phenyl ester of piperic acid (**VI**); ii. DCC/DMAP.

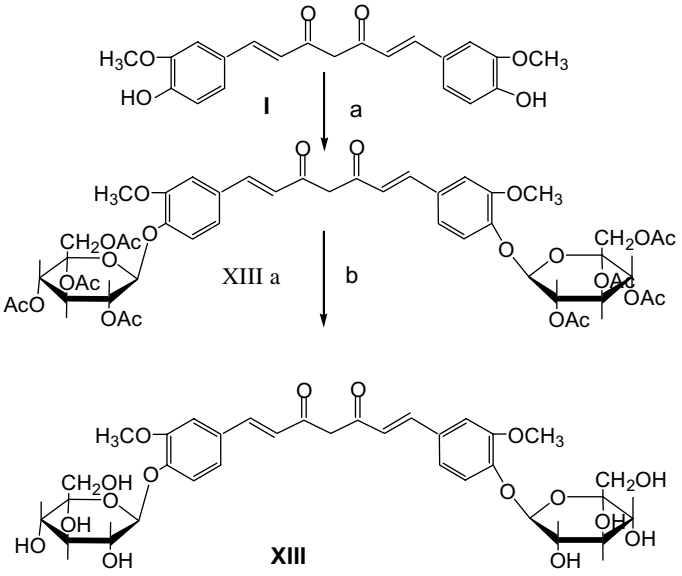
cumin was taken in pyridine and (**XII**) as colourless syrup in pyridine was added dropwise and stirred for 5 h. The reaction mixture was poured in crushed ice and extracted with EtOAc (Schemes 4 and 5). The deacetylation was done using methanolic ammonia (1:1 v/v). This reaction was followed on TLC and was found to be complete in 2 h. The di-*O*-acetyl derivative (**XIV**) of curcumin was prepared by the acetylation of both the phenolic functions of curcumin with acetic anhydride (Scheme 1).

These derivatives of curcumin viz. 4,4′-di-*O*-(glycinoyl)-curcumin (**II**), 4,4′-di-*O*-(D-alaninoyl)-curcumin (**III**), 4,4′-di-*O*-(glycinoyl-di-*N*-piperoyl)-curcumin (**VIII**), 4,4′-di-*O*-(piperoyl) curcumin (**X**), curcumin-4,4′-di-*O*-β-D-glucopyranoside (**XIII**), 4,4′-di-*O*-acetyl-curcumin (**XIV**) and the piperic acid derivative viz. piperoyl glycine (**VII**), were characterised by spectra UV, ^{1}H NMR and elemental analysis.

The antibacterial activity of curcumin-bioconjugates **II**, **III**, **VII**, **VIII**, **X**, **XIII** and **XIV**, was compared with curcumin (**I**), piperine (**IV**) and piperic acid (**V**) itself from



Scheme 4. Synthesis of D-glucose-penta acetate (**XI**) and 1-α-chloro-2,3,4,6 tetraacetyl-D-glucose (**XII**): (a) i. Dry pyridine/acetic anhydride/0 °C; ii. DMAP; (b) i. dry dichloroethane/dry HCl/0 °C; ii. Stirred at 0 °C/2.5 h.



Scheme 5. Synthesis of curcumin-4,4′-di-β-D-glucopyranoside (**XIII**): (a) **XII**/pyridine; (b) NH_3–MeOH (1:1 v/v)/2 h at rt.

known micro dilution broth susceptibility test method. The stock solutions of conjugates viz. **II**, **III**, **VII**, **VIII**, **X**, **XIII**, **XIV** along with curcumin (**I**) piperine (**IV**) and piperic acid (**V**) were prepared in acetone. The stock solution of each of these bioconjugates was serially diluted (20; 10, 5, 2.5, 1.25 μM/mL) and added to Muller–Hinton broth, after which a standardised bacterial suspension was added.

The lowest concentration of curcumin bioconjugate in μM/mL that prevented in vitro growth of microorganism has been represented as MIC (minimum inhibitory concentration) shown in (Table 2) and correlated with zone of inhibition (Table 1).

Susceptibility test in vitro was done on multiresistant bacteria specially causing secondary infections in human being, for example, *Escherichia coli*, *Staphylococcus aureus*, *Pseudomonas aeruginosa*, *Pseudomonas pyocyanin*, *Enterobactor cloacae*, *Klebsiella aeurogenus*, *Staphylococcus epidermidis*, *Staphylococcus saprophyticus*, *Micrococcus*, *Enterobactor aerogen* and *Enterococus*. These pathogens were selected for in vitro investigation along with some other Gram-positive and Gram-negative bacteria. The results have been tabulated (Tables 1 and 2).

Each test was performed in triplicate and the MICs reported represent the result of at least two repetitions. Five bioconjugates (**II**, **VII**, **VIII**, **X** and **XIV**) show good positive results on multiresistant microorganisms. The most encouraging result was found against *P. aeruginosa* with **VIII** and **X** and *E. coli* with **XIV** having MIC 2.5 μM/mL and compared with one of the best marketed antibiotic viz. *Cefepime*, which show MIC 7.0 μM/mL (6.0 μM/mL reported) showing that the di-*O*-(glycinoyl-*N*-di-piperoyl)-curcumin (**VIII**) and curcumin diacetate (**XIV**) is 2.8 times more effective than *Cefepime* at the same concentration.

The result of zone of inhibition is also encouraging as the disc containing 30 μg of *Cefepime* was purchased and same amount was loaded on separate disc, the zone of inhibition of *Cefepime* was 18 mm while (**VIII**) and (**XIV**) shows zone of inhibition, respectively, 27 and 24 mm. The MIC of II, III, VII and **XIII**, curcumin derivative were found between 5 and 10 μm/mL in most cases as compared to available antibiotics in market having MIC in between 6 and 10 μM/mL. The piperoyl curcumin (**X**) has a significant result against *P. aeruginosa* having MIC 2.5 μM/mL. In comparison to above results curcumin, piperin and piperic acid were found to be resistant in most cases. Curcumin shows MIC 20 μM/mL against *E. coli* and *S. aureus*. Piperine and piperic acid show activity at relatively high concentration.

The results suggest that positive results with **II**, **III**, **VII** and **VIII** may be due to the reason that amino acids gly-

Table 1. Antibacterial activity of curcumin bioconjugates (numerals show size of zone of inhibition in mm) of different dilutions of bioconjugates (20, 10, 5, 2.5 μM/mL) against bacterial strains

Name of bacteria	I	II	III	IV	V	VII	VIII	X	XIII	XIV
E. coli	10	—	12	—	—	10,9	12, 10	10, 8	14, 12	24, 22 18, 15
S. aureus	10	12, 10, 8	10, 8	—	—	—	10, 8	14, 12, 8	14, 12	8
P. aeruginosa	—	15, 12	—	12	—	16, 13, 8	27, 24, 20, 18	18, 15, 12, 8	—	—
P. pyocynin	—	10, 8	12, 10, 8	—	—	12, 8	16, 14, 12, 9	13, 10, 7	—	—
E. cloaca	—	12	—	10	—	12	15, 13	14, 12	—-	14, 12, 9
K. aeurogenus		—	—	10, 8	8	—	—	14, 10	16, 12, 10	—
S. epidermidis	11, 9	14, 10, 8	15, 12	8	10	12, 9	14, 9	12, 10	—	11, 8
S. saprophyticus	—	14, 12, 10	12, 10, 8	—	—	12, 8	14, 10, 8	11, 9	12, 8	12
Micrococcus	—	15, 13, 10	14, 10, 8	—	—	—	12, 9	15, 12, 10	10, 8	—
E. aerogen	—	—	—	—	—	—	—	11, 9	10	—
Enterococus	14	—	11	—	16, 12	—	14, 12, 8	—	—	11

(—) Resistant.

Table 2. Minimum inhibitory concentration (**MIC**), correlation diagram (in μM/mL) of bioconjugates against bacterial strain

Name of bacteria	I	II	III	IV	V	VII	VIII	X	XIII	XIV	*Cefeprime*
E. coli	20	—	20	—	—	10	10	10	10	2.5	18
S. aureus	20	5	10	—	—	—	10	5	10	20	20
P. aeruginosa	—	10	—	20	—	5	2.5	2.5	—	—	12
P. pyocynin	—	10	5	—	—	10	2.5	5	—	—	8
E. cloacae	—	20	—	20	—	20	10	10	—	5	18
K. aeurogenus	—	—	—	10	20	—	—	10	5	—	12
S. epidermidis	10	5	10	20	20	10	10	10	—	10	8
Staphylococcus saprophyticus	—	5	5	—	—	10	5	10	10	20	10
Micrococcus	—	5	5	—	—	—	10	5	10	—	
E. aerogen	—	—	—	—	—	—	—	10	20	—	
Enterococus	20	—	20	—	10	—	5	—	—	20	

(—) Resistant.

cine and D-alanine are natural components of bacterial cell wall. There appears to be very significant inhibition of growth in case of *P. aeruginosa* and *P. pyocyanin*. It has been reported in literature that piperine enhances the bioavailability[21] and thus the activity of many drugs as well as herbal preparations is increased. Since, the diglycinoyl curcumin was found to be relatively more active against Gram positive as well as Gram-negative bacteria vis-à-vis curcumin, it could be concluded that covalent linking of one or two glycine units with curcumin enhance its bioavailability.[9,10] This may be due to the easy recognition of glycine by the bacterial cells, which help in better cellular uptake and thereby enhances its active concentration inside the cell. Moreover, the metabolism of these conjugates is also expected to be slower than curcumin itself. In order to increase its effectiveness, it was condensed further with 2 mol of piperic acid, via biocompatible amide links. The highly positive result obtained specially with *P. aeruginosa* and *P. pyocyanin* are suggestive of its enhanced bioavailability. Similarly curcumin linked with piperic acid directly, by the reaction of curcumin and piperoyl chloride had enhanced activity. Thus the most notable result is that this novel prodrug is relatively more effective towards Gram-negative bacteria as compared to Gram positive, whereas the positive result of (**VIII**) is due to the fact that piperic acid when linked to curcumin diglycinoyl conjugate, its activity increases due to it's enhanced bioavailability.

The many fold enhancement of antibacterial activity of curcumin-di glycinoyl conjugate (**II**) on further linking it covalently with piperic acids (**V**) is quite significant. In a physical mixture of two spice components, that is, curcumin and piperine the bioavailability increases and latter may be acting as an adjuvant. However, a possible explanation that can be given is that the covalent linkage of the two compounds results in extension of the chromophores containing conjugate double bonds, thus resulting in a better binding site for receptors. However, more work is required for this verification.

The curcumin diacetate shows best results against *E. coli* with MIC (2.5 mμ/mL). Acetyl groups are attached with curcumin by ester bonds, which are biodegradable linkage, and are easily cleaved under enzymatic conditions to release curcumin at the site.

However, this approach of combining different active molecules present in nature, resulting in enhancement of their bioavailability, are certainly encouraging. These bioconjugates of curcumin are more hydrophilic than the parent molecule. The hydrophilic nature of bioconjugates may also help in their active transport across the cellular membrane.

Literature survey however suggests that neither curcumin nor any of its bioconjugates have so far been evaluated for their antifungal activity. We have now

evaluated the anti fungal activity of curcumin and its bioconjugates against specific fungi viz. *Aspergillus fumigatus*, *Candida krusei GO3*, *Candida glabrata*, *Candida albicans* (yeast), in vitro. These fungi specially cause nail infections called 'onychomycosis'. Among the onychomycosis toenail infection is more prevalent than fingernail. These fungus were obtained from a skin clinic centre from the patients diagnosed for onychomycosis on potato dextrose agar without the presence of antibiotics.

From the results shown in Table 3 we can say that curcumin piperic acid (**X**) 4,4′-(di-*O*-glycinoyl-di-*N*-piperoyl) curcumin (**VIII**) and piperoyl glycine (**VII**) show better antifungal activity specially against *C. albicans* (yeast) than curcumin (**I**), piperine (**IV**) and piperic acid (**V**) itself. The better result is probably due to the fact that the cellular uptake increases through the fungal cell wall. Though our work is still in its infancy and includes limited number of both, that is, causative organism and bioconjugates nevertheless the significance of work cannot be ignored. It opens a new era for exploring suitably designed curcumin bioconjugates as potential antibacterial/antifungal drugs. Exhaustive work based on the designing and testing the conjugates according to the molecular organisation of several pathogens is required before any definite conclusion can be reached.

The antifungal activity of curcumin bioconjugates was compared with the standard antifungal drugs like fluconazole, whereby it was found negligible in some cases (e.g., *C. krusei GO3*) and better in case of *C. albicans* (yeast). This result is quite encouraging since antifungal drugs are quite rare, and fungal infections are known to be stubborn.

To develop a new armory of agents active against antibiotic-resistant bacteria, nonconventional antibiotics could be designed that can internalise themselves in the bacterial cell wall easily. The structures should be devoid of the β-lactam ring for better efficacy.

3. Conclusion

The different derivatives of curcumin have been prepared by covalently linking it with different ligands through its two phenolic groups. All the covalent bonds used are biodegradable, that is, hydrolysable with common enzymes present in living system. This makes these derivatives as potent prodrugs, which can get hydrolysed at the target sites. The moieties selected, e.g., glycine, D-alanine, glucose and acetate are such molecules, which are well, recognises by the bacterial/fungal cells, since these are essential components of these pathogens, as well as the host cells. Therefore, these synthetic molecules are designed to be internalised with the cellular components, in order to facilitate the cellular uptake through receptor mediated endocytosis. D-Alanine is an essential component of many bacterial cell wall, while in eukaryotic cells it is always present in its L-form. Piperic acid, an essential component of the alkaloid piperine has been used, in order to enhance the bioavailability of curcumin. Thus, the combination of two active spice ingredients, that is, curcumin and piperine is likely to enhance the bioavailability and the latter may act as adjuvant.

During the present work we have concluded that the bioconjugates synthesised from curcumin are more potent than curcumin itself against many common strains of bacteria as well as fungi. Conventionally, antibiotics are used to kill bacteria or to inhibit their division with the intention of preventing replication of bacterial genome, but over use of antibiotics in human and livestock has led to the rapid evolution of mutant species of bacteria that are resistant to multiple drugs[46] and recently there has been a call for worldwide use of antibiotics rotation scheme to conquer the problems of resistance.[47] The best example is of penicillin, which had been a wonder antibiotic drug for over half a century. β-Lactamase present in bacterial cellular fluid hydrolyses the amide bond of the β-lactam ring of penicillin and cephalosporins, producing acidic derivatives, which have no antibacterial properties, thereby making the bacteria resistant to that particular antibiotic drug.

The present work suggests that there is need to develop nonantibiotic drugs,[48] which may overcome antibiotic resistance. Such highly bioactive drugs preferably herbal preparations with least toxicity (probiotics) may be in the offing. We have succeeded in our effort to a limited extent. However, such a study needs exhaustive work, that is, designing and testing of conjugates according to the specificity of the concerned pathogenic microorganism specifically its interaction with different host cells (biodiversity). A little structural variation can cause immense difference in the activity of the drug. This approach can open new vistas in the chemotherapy of infective diseases. Since these drugs can internalise themselves in the bacterial/fungal cell wall easily and their structure is also devoid of β-lactam ring or other

Table 3. Antifungal activity of curcumin bioconjugates (zone of inhibition in mm) of bioconjugates against fungal strains

Name of fungi	I	II	III	IV	V	VII	VIII	X	XIII	XIV	Fluconazole
A. fumigatus	—	—	—	—	—	—	—	—	—	—	—
C. krusei GO3	19	—	—	—	—	—	—	—	—	—	19
C. glabrata	—	—	—	—	—	—	—	—	—	—	15
C. albicans (yeast)	15	—	—	—	—	19	24	—	—	—	29

(—) Resistant.

Curcumin (**I**); 4,4′-(di-*O*-glycinoyl)-curcumin (**II**); 4,4′-(di-*O*-alaninoyl)-curcumin (**III**); piperine (**IV**); piperic acid (**V**); piperoyl glycine (**VII**); 4,4′-(di-*O*-glycinoyl-di-*N*-piperoyl)-curcumin (**VIII**); 4,4′-(di-*O*-piperoyl)-curcumin (**X**); curcumin-4,4′-(di-*O*-β-D-glucopyranoside) (**XIII**); 4,4′-(di-*O*-acetyl)-curcumin (**XIV**).

sensitive chromophores, these can prove to be highly potent β-lactamase resistant drugs.

More such bioconjugates may be designed by keeping one of the components common to either the bacterial cell wall or bacterial genome conjugated with curcumin, thereby improving the efficiency to curcumin comprehensively at low doses and may prove to be better systemic drug by concentrating mainly on and around malignant and ill-fated cells by recognizing the bacterial cells. The results prove once again the efficacy of curcumin and its bioconjugates as antibacterial and antifungal agents.

4. Experimental procedures

4.1. General

All solvents used were purified and distilled prior to use. Curcumin, glycine, alanine and piperine were purchased from Merck-Schuchardt, Germany. UV–visible spectra were recorded on Hitachi 220S UV–visible spectrophotometer. Synthetic grade solvents used were purchased from Qualigens and were purified and dried prior to use. ^{1}H NMR were recorded on DRX300 instrument.

The Mueller–Hinton broth, Agar and sterile discs were purchased from Hi Media Laboratory Ltd., Mumbai, India. Muller–Hinton broth M391 and agar M173 have been selected for testing aerobic and facultative anaerobic bacterial isolates for fastidious organisms such as *Streptococci* and *Peptococci* the agar was supplemented with 5% defibrinated blood. The microsusceptibility test were standardised at pH 7.4, agar-broth were incubated in an ambient air incubator at 37 °C.

The inoculum was prepared from broth culture that has been incubated for 4–6 h, when growth was considered in the logarithmic phase. *Cefepime* (an antibiotic drug) taken as standard. *Fluconazole* was taken as a standard anti fungal drug. The microorganisms were obtained from a city clinic from the diagnosed patients, specially a dermatophyte, nondermatophyte and yeast were isolated from the nail of patients diagnosed for onychomycosis on potato-dextrose agar without antibiotic for testing isolates, these were subcultured on sabourad dextrose agar slant and incubated at 30 °C for 10–12 days. The sterile discs with 6 mm diameter were further sterilised and charged with **II**, **III**, **IV**, **V**, **VII**, **VIII**, **X**, **XIII** and **XIV** as per requirement. After drying the discs, they were stored at 4 °C.

1,7-Bis (4-*O*-glycinoyl-3-methoxy phenyl)-1,6-heptadiene-3,5-dione (**II**) was prepared as reported.[9]

4.2. 1,7-Bis (4-*O*-D-alaninoyl-3-methoxy phenyl)-1,6-heptadiene-3,5-dione (III)

Curcumin (**I**) (368 mg; 1 mmol) was taken in 10% NaOH and mixed with *N*-phthaloylalaninoyl chloride (713 mg; 3 mmol) slowly at 0 °C with constant stirring at rt for 5 h. The completion of reaction was monitored on TLC (AcOEt–cyclohexane–EtOH: 55:40:5). The r.m. was treated with NH_3–pyridine (9:1 v/v) for 1 min at rt to remove phthaloyl group. The reaction mixture was poured in crushed ice and lyophilised to concentrate the solvent and then the product was extracted with ethylacetate, evaporated in vaccuo and purification was done by column chromatography using DCM–MeOH gradient, Yield 84% (0.448 g). Anal. Found: C, 65.49; H, 5.63; N, 5.20 calcd, for $C_{29}H_{30}O_8N_2$; C, 65.14; H, 5.65; N, 5.24. ^{1}H NMR ($CDCl_3$) δ = 3.14 (d, 6H, –C*H*$_3$); 3.81 (s, 6H, –OC*H*$_3$); 4.12 (s, 2H, C_4–*H*); 4.43–4.57 (m, 2H, 2 °C of alanine); 6.55 (d, 2H, C_2–*H* and C_6–*H*); 6.83–7.11 (m, 6H, Ar–*H*), 7.53 (d, 2H, C_1–*H* and C_7–*H*).

4.3. Piperic acid from piperine (V)

Piperine (**IV**) (2.853 g; 0.01 mol) was refluxed with ethanolic KOH (2 N, 10 mL) for 2 h. Ethanol was distilled under reduced pressure, solution cooled in ice salt bath, the solid potassium salt of piperic acid was suspended in hot water and acidified with hydrochloric acid, yellow precipitate was collected, washed with cold water and recrystallised from ethanol to yield crystals. Yield 88% (1.91 g); R_f 0.30 (DCM–MeOH: 9.5:0.5); mp 215 °C; UV λ_{max} (MeOH): 255, 335 nm; Anal. Found: C, 66.05; H, 4.56 calcd, for $C_{12}H_{10}O_4$; C, 66.04; H, 4.61. ^{1}H NMR ($CDCl_3$) δ ppm: 5.64 (d, 1H, –C*H*=CH–C=O); 5.93 (s, 2H, O–C*H*$_2$–O); 6.38 (d, 1H, –C*H*=C); 6.58–7.78 (m, 4H, olefinic and aromatic); 6.73 (d, 1H, *J* = 9, Ar–*H*); 7.25–7.51 (d, 1H, CH=C*H*=C=O); 11.2 (s, 1H, *H*O–C=O).

4.4. *p*-Nitro phenyl ester of piperic acid (VI)

To piperic acid (**V**) (2.180 g; 10 mmol) dissolved in dry dioxane (5 mL), *p*-nitro phenol (1.52 g; 11 mmol) dissolved in dioxane was added drop wise. The reaction mixture was made basic by addition of 0.5 mL pyridine and 0.5 mL TEA. After stirring for 10 min dicyclohexyl carbodiimide (DCC) (2.579 g; 12.5 mmol) was added. The reaction mixture was stirred for 2 h under nitrogen atmosphere (caution? protection from moisture) and monitored on TLC. The completion of reaction was assessed by total consumption of starting reagent. R_f = 0.58 (DCM–MeOH: 9.5:0.5).

4.5. Piperoyl glycine (VII)

Glycine (0.15 g; 2 mmol) was dissolved in dry pyridine (5 mL) was added drop wise to above activated ester of piperic acid (**VI**) (0.748 g; 2.2 mmol). The r.m. was stirred for 10–20 min and DCC (515 mg; 2.5 mmol), DMAP (12.2 mg; 0.1 mmol) were further added. The r.m. was stirred for 3 h and monitored on TLC. The precipitate of dicyclohexyl urea (DCU) started appearing after 30 min and completed after 3 h. The reaction mixture was filtered to separate DCU and filtrate was evaporated to half of its volume and then poured in 2% sodium bicarbonate solution (to neutralise the residual acid and to separate excess of *p*-nitro phenol) and extracted with dichloromethane (4 × 10 mL). Organic layer was collected and further washed with distilled

water, dried over anhyd Na_2SO_4 and filtered. The combined dichloromethane layer were concentrated and crystallised with dry ethanol to get desired product. Yield 70% (0.192 g); R_f 0.39 (DCM–MeOH:: 9.5:0.5); mp 232 °C; UV λ_{max} (MeOH): 300, 340 nm.; Anal. Found: C, 62.05; H, 4.75; N, 5.08 calcd, for $C_{14}H_{13}NO_5$; C, 61.08; H, 4.76; N, 5.08. ^{1}H NMR ($CDCl_3$) δ ppm: 3.90 (d, 2H, C*H*$_2$–C═O); 5.70 (d, 1H, –C*H*═CH–C═O); 5.86 (t, 1H, CO–N*H*); 5.96 (s, 2H, O–C*H*$_2$–O); 6.72–7.86 (m, 4H, olefenic and aromatic); 6.90 (d, 1H, $J = 9$, Ar–*H*); 7.21–7.48 (d, 1H, CH═C*H*═C═O).

4.6. 1,7-Bis-[(4-*O*-glycinoyl-*N*-piperoyl)-3 methoxy phenyl] 1–6-heptadiene-3,5-dione (VIII)

Di-glycinoyl curcumin (**II**) (0.482 g; 1 mmol) dissolved in dry pyridine (5 mL) and solution added drop wise to the above activated ester (**VI**) (0.748 mg; 2.2 mmol). The r.m. was stirred for 10–20 min and DCC (257 mg; 1.25 mmol), DMAP (12.2 mg; 0.1 mmol) were further added. The r.m. was stirred for 3.5 h and monitored on TLC. The precipitate of dicyclohexyl urea (DCU) started appearing after 30 min and completed after 3 h. The r.m. was filtered to separate DCU and filtrate was evaporated to half of its volume and then poured in 2% sodium bicarbonate solution (to neutralise the residual acid and to separate excess of *p*-nitro phenol) and extracted with dichloromethane (4×10 mL). Organic layer was collected and further washed with distilled water, dried over anhyd Na_2SO_4 and filtered. The combined dichloromethane layer were concentrated and residue crystallised in dry alcohol to get desired product. Yield 48% (0.423 g); R_f 0.52 (DCM–MeOH:: 9.5:0.5); UV λ_{max} (MeOH): 240, 298, 340 nm.; Anal. Found: C, 66.59; H, 4.79; N, 3.15 calcd, for $C_{49}H_{42}N_2O_{14}$; C, 66.65; H, 4.79; N, 3.17. ^{1}H NMR ($CDCl_3$) δ ppm: 3.65 (s, 6H, –OC*H*$_3$); 4.15 (s, 2H, C$_4$–*H*); 4.65 (d, 2H, O═C–C*H*$_2$); 5.65 (d, 1H, –C*H*═CH–C═O); 5.91 (s, 2H, O–CH$_2$–O); 6.51 (t, 1H, CH$_2$N*H*═C═O); 6.63 (d, 2H, C$_2$–*H* and C$_6$–*H*); 6.72–7.80 (m, 4H, olefenic and aromatic); 6.82–7.15 (m, 6H, Ar–*H*); 6.90 (d, 1H, $J = 9$, Ar–*H*); 7.18–7.48 (d, 1H, CH═C*H*–C═O); 7.48 (d, 2H, C$_1$–*H* and C$_7$–*H*).

4.7. Piperoyl chloride (IX)

Piperic acid (**V**) (2.18 g, 0.01 mol) was heated on water bath and to it was added redistilled thionyl chloride (1.09 mL, 0.015 mol) during 45 min, shook the flask from time to time to ensure mixing. Refluxed for 30 min, protected from moisture and distilled the product at reduced pressure. Yield 65% (1.539 g), mp 144–148 °C.

4.8. 1,7-Bis-[(4,4′-di-*O*-piperoyl)-3 methoxy phenyl] 1–6-heptadiene-3,5-dione (X)

Curcumin (**I**) (0.736 g; 2 mmol) was taken in dry pyridine and mixed with piperoyl chloride (**IX**) (1.065 g; 4.5 mmol), stirred at rt for 6 h. After the completion of reaction as indicated by TLC the reaction mixture was poured into crushed ice and repeatedly extracted with EtOAc. The organic layer was concentrated and purified by silica gel column chromatography using DCM–methanol gradient. Yield 48% (0.737 g); UV λ_{max} (MeOH): 230, 295, 390 nm; R_f: 0.87 (MeOH–DCM:: 0.5:9.5). ^{1}H NMR ($CDCl_3$) δ ppm: 3.65 (s, 6H, –OC*H*$_3$); 4.15 (s, 2H, C$_4$–*H*); 4.65 (d, 2H, O═C–C*H*$_2$); 5.65 (d, 1H, –C*H*═CH–C═O); 5.91 (s, 2H, O–CH$_2$–O); 6.51 (t, 1H, CH$_2$N*H*═C═O); 6.63 (d, 2H, C$_2$–*H* and C$_6$–*H*); 6.72–7.80 (m, 4H, olefenic and aromatic); 6.82–7.15 (m, 6H, Ar–*H*); 6.90 (d, 1H, $J = 9$, Ar–*H*); 7.18–7.48 (d, 1H, CH═C*H*–C═O); 7.48 (d, 2H, C$_1$–*H* and C$_7$–*H*).

4.9. 1,2,3,4,6-Penta-*O*-acteyl-α-D-glucopyranose (XI)

This was prepared by standard protocol.[44]

4.10. 1-α-Chloro-2,3,4,6-tetra-*O*-acetyl-glucopyranose (XII)

This was prepared from acetylated glucose (**XI**) (2 g; 5.12 mmol) by dissolving in cold (0 °C) dry dichloromethane (12 mL) and saturating the solution with anhydrous hydrogen chloride over 2.5 h at 0 °C. The solution was removed in vacuo (bath temperature, 25 °C). Colourless syrup was crystallised with diethyl ether. Yield 68% (1.276 g), R_f 0.66 (DCM–MeOH:: 9:1), mp 98–101 °C. ^{1}H NMR ($CDCl_3$) 1.95–2.09 ($4 \times$ br s, 12H) acetyl *H*′s; 4.05–4.10 (d, 2H) *H*6a′ and *H*6b′; 4.41–4.46 (m, 1H) *H*5′; 5.00 (dd, $^3J_{2\ni-1\ni} = 3.9$ Hz, $^3J_{2\ni-3\ni} = 10.6$ Hz, 1H) *H*2′; 5.36 (dd, $^3J_{3\ni-2\ni} = 10.6$ Hz, $^3J_{3\ni-4\ni} = 3.2$ Hz, 1H) *H*3′; 5.45–5.48 (m, 1H) *H*4′; δ 6.65 (d, $^3J_{1\ni-2\ni} = 3.9$ Hz, 1H) *H*1′.

4.11. 4,4′-Di-*O*-β-D-glucopyranocurcumin (XIII)

Curcumin (**I**) (0.368 g; 1 mmol) was taken in dry pyridine and the solution of (**XII**) (0.806 g; 2.2 mmol) in pyridine was added drop wise in a ice bath. The r.m. was removed from ice bath and stirred at rt for 3 h. Reaction was monitored on TLC. After the completion of reaction mixture was poured in crushed ice and repeatedly extracted with EtOAc. The organic layer was concentrated and treated with (6 mL) ammonia–methanol (1:1 v/v) for 2 h at rt to hydrolyse the acetyl groups. The reaction was followed on TLC. Poured in crushed ice and extracted with EtOAc. The organic layer was concentrated and purified by silica gel column chromatography using DCM–MeOH. Yield 46% (0.318 g). Anal. Found: C, 57.21; H, 5.73 calcd, for $C_{33}H_{40}O_{16}$; C, 57.21; H, 5.83. ^{1}H NMR ($CDCl_3$) δ ppm = 3.85 (s, 6H, –OC*H*$_3$); 4.02–4.10 (m, 2H) *H*6a× and *H*6b×; 4.29–4.33 (m, 1H) *H*5×; 5.13 (s, 2H, C$_4$–*H*); 5.29–5.31 (m, 2H) *H*3c and *H*4×; 5.46–5.47 (m, 1H) *H*2×; 6.48 (d, 2H, C$_2$–*H* and C$_6$–*H*), 6.85–7.08 (m, 6H, Ar–*H*), 6.93 (br s, $^3J_{1\ni-2\ni} = 1.2$ Hz, 1H) *H*1×; 7.52 (d, 2H, C$_1$–*H* and C$_7$–*H*).

4.12. 1,7-Bis-4,4′-[(di-*O*-acetyl)-3-methoxy phenyl] 1–6-heptadiene-3,5-dione (XIV)

To curcumin (**I**) (0.368 g; 1 mmol) dissolved in 10% aqueous sodium hydroxide and 7.0 g of crushed ice

was added followed by 0.18 mL (2.6 mmol) of acetic anhydride and shook vigorously for 5 min. Stirred for 2 h, and extracted with carbon tetrachloride. Concentrated the organic layer in vacuo. Organic layer was washed with 1% $NaHCO_3$ until effervescence ceased. Dried the organic layer over Na_2SO_4. Crystallised from absolute ethanol. Yield 74% (0.334 g), mp 160 °C. ^1H NMR ($CDCl_3$) δ ppm = 3.75 (s, 6H, –OCH_3), 4.10 (s, 2H, C_4–*H*), 4.17 (s, 6H, O–C–*O*–CH_3), 6.45 (d, 2H, C_2–*H* and C_6–*H*), 6.81–7.17 (m, 6H, Ar–*H*), 7.44 (d, 2H, C_1–*H* and C_7–*H*).

4.13. To determine zone of inhibition by Kirby–Bauer's method[9]

The antibacterial susceptibility test was done by determining zone of inhibition by Kirby–Bauer's method.[45] The curcumin bioconjugates, curcumin, curcumin-piperic acid conjugate and piperic acid itself were weighed and dissolved in acetone to make a solution of concentration 120 μM/mL. From this stock solution serial dilution have been done to 20, 10, 5, 2.5 and 1.25 μM/mL with acetone in sterile test tubes. Sterilised filter discs were dipped in these solutions and subsequently dried to remove acetone. Mueller–Hinton agar was prepared and allowed to solidify. One of these discs was kept free from antibiotic and served as growth control. Twelve different bacteria were selected viz.—*Escherichia coli*, *S. aureus*, *P. aeruginosa*, *Pseudomonas pyocynin*, *E. cloacae*, *K. aeurogenus*, *Staphylococcus epidermidis*, *S. saprophyticus*, *Micrococcus*, *E. aerogen* and *Enterococus* and 1 mL of each bacterial culture broth were added in the Mueller–Hinton plates and spread with the help of sterile spreader. The filter paper discs soaked in above mentioned dilutions of curcumin, piperic acid and their conjugates were placed aseptically over the inoculated plates using sterile forceps. The plates were incubated at 37 °C for 18 h, in upright position. The zone of inhibition was measured using scale (Table 1).

4.14. To determine MIC by the micro dilution broth susceptibility test

Different concentrations 20, 10, 5, 2.5 and 1.25 μM/mL of the compound were prepared in sterile dry test tubes to determine minimum inhibitory concentration (MIC). Mueller–Hinton broth (M391) was prepared and 4.9 mL of it was taken in each test tube and were sterilised after plugging. After cooling 0.1 mL of each dilution were added to the test tubes and the final volume was made up to 5.0 mL. To each of test tube 0.1 mL of bacterial culture broth was added. The test tubes were shaken to uniformly mix the inoculum with the broth. The tubes were incubated at 37 °C for 18 h. Appearance of any turbidity shows that the compound is not able to inhibit the growth of the bacteria, while no turbidity indicates the inhibition of microorganism by the sample (Table 2).

4.15. In vitro-antifungal test[49]

For antifungal testing the curcumin bio-conjugates, curcumin, piperine and piperic acid solution was prepared in acetone at initial concentration 120 μM/mL and serially diluted to make effective concentration of 20, 10 and 5.0 μM/mL. The dermatophytes, nondermatophytes and yeast were isolated from nail chippings from suspected cases of onychomycosis referred from a skin clinic at Allahabad. The most prevalent causative organisms viz. *A. fumigatus*, *C. krusei GO3*, *C. glabrata*, *C. albicans* (yeast) were selected. These fungii were isolated from the nails of patients diagnosed for onychomycosis, on sabourad dextrose agar without antibiotics. For testing isolates were subcultured on subourad dextrose agar slant and incubated at 30 °C for 10 days. Sterile discs with 6 mm diameter were further sterilised and charged with compounds (**I**, **II**, **III**, **IV**, **V**, **VII**, **VIII**, **X**, **XIII** and **XIV**) and after drying these discs were stored at 4 °C.

4.16. Preparation of the inoculum[49]

A standardised inoculum was prepared by counting microconidia. Cultures were grown on soluble dextrose agar for 10 days at 37 °C sterile saline sol (0.85%) was added to the slant and culture were gently swabbed with cotton tipped applicator to dislodge conidia from the hyphal mat. The suspension was transferred to a sterile tube and volume was adjusted to 5 mL with sterile saline solution. The resulting suspension was counted with homocytometer and diluted in sabourad broth to 5 mL. Subourad dextrose agar was poured to depth of 5 mm in 90 mm petridiscs and stored at 4 °C. The plates were dried; standardised suspension was poured and uniformly spread by means of swab discs. The excess inoculum was drained.

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