Chapter I - 'In vitro' antifungal activity.

Materials and Methods

Strains of Candida used

Nine strains of Candida including five strains of C. albicans (viz: SKF, CDRI, 48-72, 9150, 3100) and one strain of each C. tropicalis (204); C. pseudotropicalis (513); C. krusei (205); and C. parakrusei (208), were used as test organisms. Strain numbers SKF, 48-72, 513, 205 and 208, were obtained from the Institute Pasteur, Paris, while 3100 and 9150 were obtained from the Microbiology Department, St. Xaviers College, Bombay. The strain (CDRI) was received from Dr. O.P. Srivastava, C.D.R.I., Lucknow.

Maintenance of strains

Pure line cultures of Candida were maintained by subculturing them bimonthly on Sabouraud agar, slants. Sabouraud medium consisted of Glucose 40 g., Bacto Peptone 10 g. Agar (Oxoid) 20 g. and Distilled water 1000 ml.

The pH of the medium remains at 5.8. The medium
was sterilized by autoclaving at 10 lbs. pressure, for 20 minutes. Before autoclaving, the medium was dispensed in 1 x 15 cm. test tubes. The tubes were kept in slanting position to make agar slants.

Test compounds

Potential antifungal compounds and standard drugs used in the present investigation were obtained from the following sources:

i) Naphthoquinones having substituents in the aromatic ring, fused heterocyclic system with 1,4-naphthoquinones and 1, 4 benzoquinones and other allied compounds were synthesised or isolated from natural products in the Medicinal Chemistry Division of C.D.R.I., Lucknow.

ii) Hibitane (Imperial Chemical Industries Ltd., London) and Dequiam (British Hydrological Corporation Research Laboratories, London) were obtained from Dr. S.R. Das, C.D.R.I., Lucknow.

iii) Antibiotic X-63 and nystatin were obtained from Dr. V.C. Vora, C.D.R.I., Lucknow.

iv) Amphotericin B was obtained from Sarabhai Merck's, Baroda.

The compounds screened are classified in six series namely: (1) Substituted 1,4-benzoquinones and its heterocyclic
analogues; (ii) Substituted 1,4-naphthoquinones;
(iii) Substituted naphthazarine (5, 8-dihydroxynaphthoqui-
nones); (iv) Substituted isoquinolines; (v) Naphthoquinones
norbornene; (vi) Substituted anthraquinones. Antibiotic
X-63 and other compounds, which do not belong to the series
cited above, have been grouped as miscellaneous.

Testing Method

Anticandidosis activity was determined by making
two-fold serial dilutions of compounds in Sabouraud liquid
medium. The compounds were suspended in 50 per cent ethanol
at a concentration of 2 mg./ml. Seeded broth was made by
adding 1.25 ml. of 18 hour growth of test organisms (in the
logarithmic phase) to 20 ml. of the medium. Thus seeded
broths contained 5 to 8 x 10^6 yeast cells per ml. The count
of cells were made in haemocytometer. Seeded broths were
dispensed into test tubes (10 x 100 mm.), so that the
first tube of each set contained 3.8 ml. and the subsequent
fifth
ubes 2 ml. One mililitre of suspension of compounds was
added to the first tube and after mixing, 2 ml. was trans-
ferred to the next tube. The process was continued and
2 ml. from the last tube was discarded. Thus all tubes
contained 2 ml. of seeded broth with various concentration
of compounds from 100 /ug./ml. to 0.03 /ug./ml. All the
tubes, including the control tubes, (without compounds) were incubated at 37°C. Minimum Inhibitory Concentration (MIC) of each compound against all the 9 strains of Candida were recorded after 24 and 48 hours of incubation by visual turbidity method. In some cases the results were recorded up to 96 hours of incubation.

Viable cell counts of serial dilutions of hibitane were compared with those of amphotericin B. Samples were withdrawn from tubes after 24, 48, 72 and 96 hours of incubation and decimally diluted into sterile 0.85% saline. Aliquots of 0.1 ml., from the diluted suspensions, were plated in triplicate on Sabouraud agar plates. These were incubated for 48 hours at 37°C and the number of yeast colonies were recorded.

Results and Discussion

The maximum level, at which potential anticandididosis compounds were tested, was MIC 100 μg./ml., as this concentration can reach in patients sera with therapeutically large doses of a potential drug. A potential anticandididosis agent with a MIC value between 1-12.5 μg./ml. has greater chance to go to the clinics after animal experiments and preclinical pharmacology.

The results of 'in vitro' testing of potential
anticandidosis agents, are presented in Table I. Compounds showing 'in vitro' activity (MIC ≥ 25 /μg./ml.), are listed in Table II. Seventeen out of 59 compounds of seven different series besides the standard drugs showed activity against 4 strains of C. albicans which were sensitive to amphotericin B (SKF, CDRI, 48-72, 9150). These are 72-375 (3.125-12.5 /μg./ml.), Epoxy arnebin-1 (6.25 - 25 /μg./ml.), 72-489 (6.25 - 12.5 /μg./ml.), 73-584 (6.25 - 25 /μg./ml.) arnebin-4 (6.25 - 25 /μg./ml.), 74-363 (6.25 - 25 /μg./ml.), antibiotic x-63 (0.78 - 3.125 /μg./ml.), deciquamine (3.125 - 6.25 /μg./ml.) and hibitane (6.25 - 12.5 /μg./ml.). The following compounds showed low or marginal activity (12.5 - 25 /μg./ml.): 74-367, 72-485, 74-60, 73-38, arnebin-3, 73-157, 74-57 and 74-58. The strain 3100, of C. albicans which was found to be resistant to 25 /μg./ml. of amphotericin B, was inhibited by compound 74-363 at 12.5 /μg./ml. Compound 72-375 and 72-489 also inhibited this strain at 25 /μg./ml.

Some naphthoquinones showed good activity against some strains of the Candida species (viz: 204, 513, 205, 208). Epoxy arnebin-1 and arnebin-4, inhibited strains 204 and 205 at 1.56 /μg./ml. Compound 72-375, inhibited strains 204, 205, 208 at 6.25 /μg./ml. Compound 72-489, showed activity against strains 205, 208 at 3.125 /μg./ml.
MIC value of other compounds (72-485, 74-60, 74-363, 73-38, arinebin-3, 73-584, 73-157 and 74-58), against these strains, were between 6.25 to 25 /μg./ml.

Amphotericin B and nystatin were tested as standard control drugs. Amphotericin B was very active against four strains of *C. albicans* (viz: SKF, CDRI, 48-72, 9150). The fifth strain 3100, was resistant against this drug as there was no visible turbidity after 24 hours of incubation in 1.56 /μg./ml., but considerable turbidity appeared in 12.5 /μg./ml., concentration, after 48 hours of incubation. When the incubation period was prolonged to 3-4 days, turbidity appeared even in 50 /μg./ml. concentration. Antibiotic X-63 was found to be equally active against all the five strains of *C. albicans* including strain 3100. Nystatin, deciquam and hibitane showed appreciable activity against nearly all strains tested. Amphotericin B showed no inhibitory action against the 4 strains of *Candida* spp. It appears that amphotericin B's range of activity was limited to only four *C. albicans* strains.

Table III shows the comparative results of hibitane and amphotericin B against 8 strains of *Candida* including four strains of *C. albicans*. The MIC were recorded daily upto 4 days of incubation. The growth of yeast settles at the bottom of the tubes and the dilution showing
inhibition was one, which did not show any deposit. In case of both hibitane and amphotericin B which are insoluble, the higher dilutions showed deposits at the bottom of the tubes even when there was no growth of yeast. To overcome this difficulty and to find out the candidical concentration the viable yeast counts of the dilutions showing inhibition were made.

Against the 3 sensitive strains of *C. albicans* (viz: SNF, 48-72 and 9150) the MIC of amphotericin B after 24 hours incubation was 0.78 µg./ml., which increased to 6.25, 3.125 and 6.25 µg./ml., respectively, when the incubation period was prolonged to 4 days. However, the MIC of hibitane against these three amphotericin B sensitive strains remained practically the same on all the days of incubation.

The strain 3100 was resistant to amphotericin B since the MIC after 24 hours was 1.56 µg./ml., by the visible growth criteria. There was no growth seen in dilutions of 1.56 µg./ml., and higher concentrations, but when the viable counts of these dilutions were made, they showed applicable number of colonies in dilutions up to 12.5 µg./ml. Though, candidostatic concentration after one day of incubation was 1.56 µg./ml.; the candidical concentration was 25 µg./ml. These two values became 100 µg./ml., after 4 days of incubation.
It is interesting to note that in case of hibitane 6.25 and 3.125 μg./ml. concentrations showed viable counts of $1.5 \times 10^2$ and $5.0 \times 10^4$ per ml., respectively after 3-day incubation. The count in the former concentration became nil while there was only a 10-fold increase in the later after 4-day incubation. This showed that both static and cidal activity after 4-day incubation remained at 6.25 μg./ml.

Against the 4 *Candida* species, amphotericin B showed no activity after 4-day incubation, though hibitane showed static and cidal activities against *C. tropicalis*, *C. pseudotropicalis* and *C. parakrusei* in the range of 3.125 - 12.5 μg./ml. Against *C. krusei* this compound showed a low activity (25 μg./ml.). Theoretically hibitane appears to be better than amphotericin B as far as the microbiological aspect of drug action is concerned.

It has been observed for many years that quinones especially benzoquinone and naphthoquinone derivatives show appreciable biological activity of various types. They also possess potent antibacterial and antifungal activities.

Recently Gershon and Shanks (1975), have reported the antifungal activity of certain 1, 4-naphthoquinones. From their results they concluded that among the active 1, 4-naphthoquinone derivatives, all possessed at least one electron releasing or weakly electron withdrawing substituent in
the 2 or 3 positions. This they explained on the basis of their enhanced hydrogen bonding capacity and allowing this toxicant to bind more strongly at its site of action. This is in agreement with the hypothesis of Ambrogi et al., (1970), who observed similar findings in the same series of compounds. Vladimirtsev et al., (1969), also observed similar findings a year earlier. Gershon and Shanks (1975), further concluded that when a strongly hydrophilic substituent like hydroxyl or carboxylic acid bisulfite radicals, were added to the naphthoquinones, less of activity occurred.

In the present studies, compound 74-367 of Series I (substituted 1-4, benzoquinones and its heterocyclic analogues) was found to have feeble or slight activity against all strains of Candida. In series II (substituted naphthoquinones), compound 72-375 (8-chlorojuglone), showed very good activity against 4 strains of C. albicans SKF1, CDRI, 48-72 and 9150 while compounds 72-485 and 74-60 showed only weak activity. This shows that chloro-substitution at 8 position (72-375), enhances the activity and could well be compared with compound 72-489 (naphthazarine) and arnebins and their derivatives (Epoxy arnebin-1, arnebin-3, and arnebin-4) which carry the 5, 8-dihydroxy groupings in the aromatic rings of 1, 4-naphthoquinones. In order to see the effect of fused ring systems to quinone ring of
1,4-naphthoquinones, norbonene (series V) and anthraquinone derivatives (series VI), were also included in the present study. The results show that 73-584 (series IV) and two compounds 73-157 and 74-58 (series VI) also were fairly active.

Compound 74-363 (Table II), showed moderate activity against amphotericin B resistant strain of C. albicans, (3100) while two compounds 72-375 and 72-489, appear to possess low activity. All other compounds tested (Table II) excluding miscellaneous group, were found to be inactive. Antibiotic X-63, deciquam and hibitane compare well with the standard drug, nystatin as regards the activity against this strain.

Conclusion

Fifty nine compounds were tested in vitro for their antifungal activity against 9 strains of Candida including 5 strains of C. albicans and one each of C. tropicalis, C. pseudotropicalis, C. krusei and C. parakrusei. These compounds were classified in seven series namely: (1) 7 substituted 1, 4-benzoquinones and their heterocyclic analogues; (2) 9 substituted 1,4-naphthoquinones; (3) 14 substituted naphthazarines (5, 8-dihydroxy
naphthaquinones); (4) 3 substituted isoquinolines; (5) 2 naphthaquinone norbernones; (6) 15 derivative of anthraquinones and (7) 9 compounds placed under miscellaneous heading including amphotericin B and nystatin as standard drugs.

Fourteen compounds belonging to series 1-6 and three of series 7 (hibitane, deciquam and antibiotic X-63) had shown activity against various strains of Candida. Compounds, 72-375 (8-chlorojuglone), epoxy arnebin-1 (2-1'-acrylate-4'-methyl-3'; 4'-epoxy pentanyl naphthazarine); 72-489 (naphthazarine), 73-157 (1,4-dihydroxy-5, 8, 8a, 10a-tetrahydro 9, 10 anthraquinone), 74-58 (1, 4-dihydroxy-5-hydroxy)-8-chloro 1,4, 4a, 9a-tetrahydro-9, 10, anthraquinone), antibiotic X-63, hibi tane and deciquam showed moderate activity against 9 strains of Candida. Few substituted derivatives of quinones (74-367, 72-485, 74-60, 74-363, 73-38, arnebin-3, arnebin-4, 73-584, 74-57), showed mild activity.

It is interesting to note that among the quinone series only compound 74-363 (ethyl, 1-ethyl-3-methyl isoquinoline-5, 8-quinone-4-carboxylate), showed slight activity against 4 amphotericin B sensitive strains of C. albicans (SKF, CDRI, 72-48 and 9150), and moderate activity against the resistant strain 3100.
Habitane had shown candistatic and candidical activities between 3.125 and 12.5 μg./ml., against 8 strains of Candida tested. It was also active against the strain of C. albicans which is resistant to amphotericin B at 6.25 μg./ml. The static and cidal activities of this compound remained practically the same even up to 4 days of incubation.
other miscellaneous compounds in Sabouraud broth at 37°C

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