Chapter 4
Solvent toxicity

4.1. Introduction

If plants are selected based on ethnomedicinal use, the extraction procedure used in folk medicine must be kept in mind. However, a search for biological activity several solvents of different polarity can be used to isolate all the possible active compounds present. Since the chemical composition of the plant is unknown, the nature of the solvent used affects the composition of the crude extract. Solvents frequently used include methanol, ethanol, acetone, water, ethyl acetate and dichloromethane or combinations thereof. Non-polar solvents yield more lipophilic components, while alcoholic solvents give a larger spectrum of a polar material (Stecher, 2003). Ethanol and water are the most widely used solvents based on hygiene and availability. However acetone is usually used in preference as solvent for extraction because it extracts polar and non-polar components from the plant material, is miscible with water, very volatile, has low toxicity in antimicrobial bioassays and is easily removed from the plant material at low temperature (Eloff, 1998a).

To quantify antimicrobial activities, extracts have to be dried. Frequently it is difficult to resolubilize extracts even in the solvent originally used. Although acetone is an excellent extractant for a wide range of polarity compounds, in our experience especially relatively polar or non-polar extracts are completely soluble in acetone. In serial dilution assays the solvent has to be miscible with water. Water frequently does not dissolve the intermediate polarity or non-polar components of a dried extract. A detergent such as Tween 80 could be added, but a detergent could be toxic to microorganisms. An alternative is to use solvents such as methanol, ethanol or dimethyl sulfoxide (DMSO). To avoid solvents affecting the toxicity of an extract, they should first be tested for any effects against the target fungi.

4.2. Method

4.2.1. Solvents used

Four solvents with different polarities were used, i.e. dimethyl sulfoxide, acetone, methanol and ethanol.

4.2.2. Bioassays

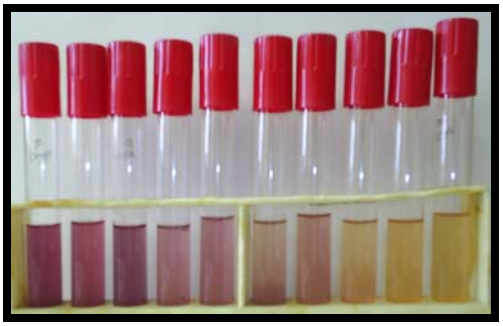
Different concentrations of DMSO, acetone, methanol and ethanol were prepared in sterilized test tubes from 10% to 100%. Dilutions of solvents were made with sterile distilled water. Fungal test organisms (**Section 5**) were prepared in Sabourand dextrose broth. One milliliter of each culture was transferred into test tubes and mixed well. Four hundred microlitres of 2 mg/ml of *p*-iodonitrotetrazolium violet (Sigma®) (INT) dissolved in water was added to each of the test tubes. Test tubes were incubated for three to five days at 35 ^{o}C at 100% relative humidity to ensure adequate colour development.

4.3. Results

Toxicity of different solvents on tested fungi was investigated using macrodilution method and *p*-iodonitrotetrazolium violet (INT) as indicator. With some fungi the differences in response to acetone were easier to notice than with other (**Figure 4.1**). Where fungal growth was inhibited, the solution in the tube remained clear or had a distinct decrease in colour after incubation with INT.

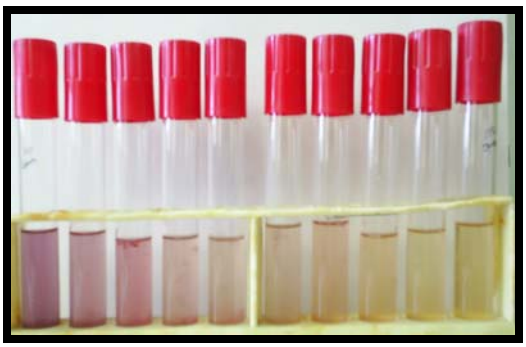
Aspergillus fumigatus

Candida albicans



Cryptococcus neoformans

Microsporum canis



Sporothrix schenckii

Figure 4.1. Test tubes of 10% to 100% acetone from left to right for each group mixed with different fungi and 2 mg/ml of *p*-iodonitrotetrazolium violet (INT) as an indicator. Purple colours indicate fungal growth and clear tubes indicate no growth.

Macrodilution assay was chosen, because it was easy to use different percentages i.e. starting from 100 to 10%. With serial microplate assay we used (Eloff, 1998) it is difficult to have values above 25%. The results of all the solvents are presented in **Table 4.1**.

Only the visual results with acetone were presented in **Figure 4.1**. The results of all the solvents are presented in **Table 4.1**.

Table 4.1. Toxicity of different solvents on tested fungi

Concentrations (%)	DMSO					Acetone					Ethanol					Methanol				
	C. albicans	*C. neoformans*	*M. canis*	*S. schenckii*	*A. fumigatus*	*C. albicans*	*C. neoformans*	*M. canis*	*S. schenckii*	*A. fumigatus*	*C. albicans*	*C. neoformans*	*M. canis*	*S. schenckii*	*A. fumigatus*	*C. albicans*	*C. neoformans*	*M. canis*	*S. schenckii*	*A. fumigatus*
10	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
20	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
30	+	+	+	+	+	+	+	+	+	+	+	+	-	+	+	+	+	-	-	+
40	+	+	+	+	+	+	+	+	+	+	+	+	-	+	+	+	+	-	-	+
50	+	+	+	-	+	+	+	+	+	+	-	+	-	-	-	+	+	-	-	+
60	+	+	+	-	-	+	+	+	+	+	-	-	-	-	-	-	+	-	-	-
70	-	+	-	-	-	-	+	-	+	-	-	-	-	-	-	-	-	-	-	-
80	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
90	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
100	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-

+ growth, - no growth

The MIC values are then calculated using the known density of 100% of the solvent. For example, if the density of acetone is 0.8 gm/ml then at 0.4 ml/ml it equals 0.4 X 0.8 g/ml = 0.32 g/ml = 320 mg/ml. From this it follows that the concentration of 100% acetone is 800 mg/ml. The MIC values were calculated from **Table 4.1** and results are presented in **Table 4.2**.

Different solvents were toxic to different fungi at different concentrations and **Table 3.2** had MIC values at which solvents kill different fungi. DMSO was toxic to *S. schenckii* in 40% (0.40 ml/ml) and *A. fumigatus* in 50% (0.50 ml/ml). *C. albicans* and *M. canis* can still survive in 60% (0.60 ml/ml), but *C. neoformans* can survive in 70% acetone. Among the tested solvents acetone was found not to be toxic to fungi tested, as they can all survive in concentrations of 60% to 70%. Methanol was relatively toxic to *M. canis* and *S. schenckii*, both at 20% and ethanol was toxic to *M. canis* at 20%.

Table 4.2. MIC values and equivalent concentrations of different solvents against tested fungi

Microorganisms	MIC values (mg/ml) and % final concentration				
	DMSO	**Acetone**	**Ethanol**	**Methanol**	**Average**
C. albicans	660 (60%)	474 (60%)	324 (40%)	395 (50%)	**465**
C. neoformans	770 (70%)	553 (70%)	405 (50%)	474 (60%)	**553**
M. canis	660 (60%)	474 (60%)	162 (20%)	158 (20%)	**365**
S. schenckii	440 (40%)	553 (70%)	324 (40%)	158 (20%)	**370**
A. fumigatus	550 (50%)	474 (60%)	324 (40%)	395 (50%)	**438**
Average	**616**	**512**	**304**	**320**	

On average DMSO is by far the least toxic of all the solvents tested followed by acetone. *C. neoformans, C albicans* and *A. fumigatus* survived under higher concentrations of methanol (**Figure 3.2**). The average MIC of DMSO on all tested fungi was 616 mg/ml, followed by acetone with 512 mg/ml, methanol with 304 mg/ml and ethanol with 320 mg/ml (**Figure 3.3**).

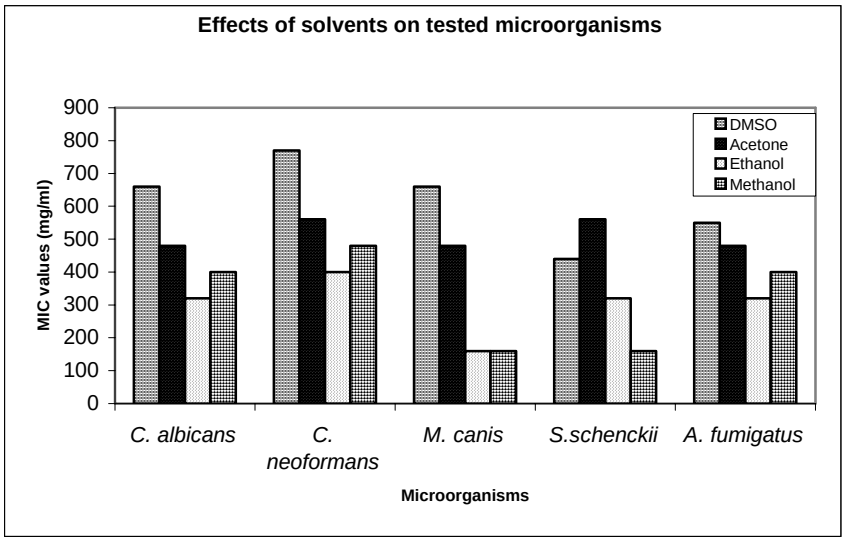


Figure 4.2. Effects of solvents on tested fungi

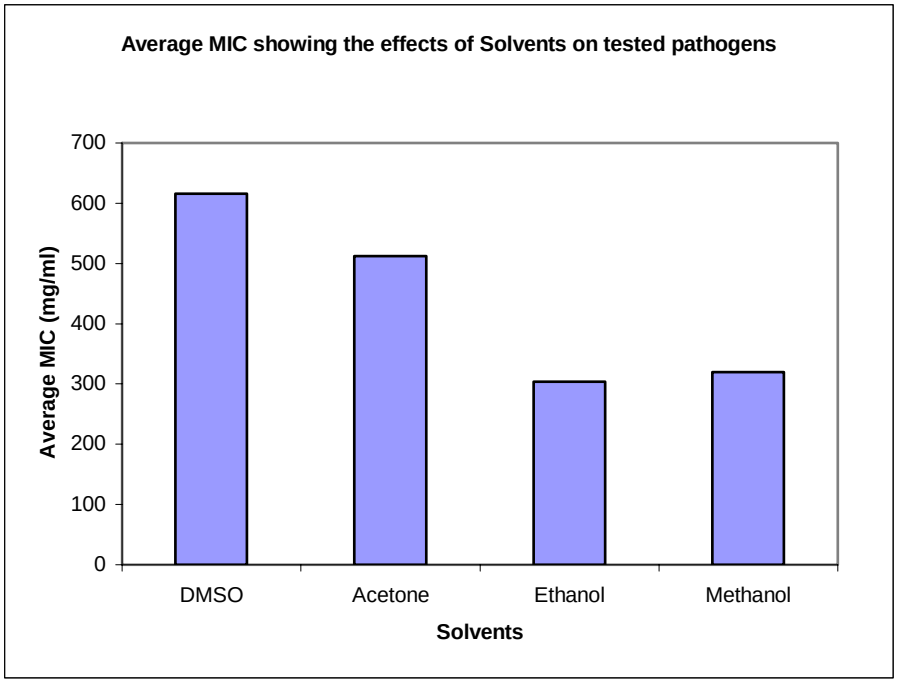


Figure 4.3. Average MIC showing the effects of solvents on tested fungi

C. neoformans and *C. albicans* were resistant to solvents, with an average MIC of 553 and 465 mg/ml respectively, followed by *A. fumigatus* (436 mg/ml). *M. canis* and *S. schenckii* were very sensitive with an average MIC of 365 and 370 mg/ml respectively (**Figure 4.4**).

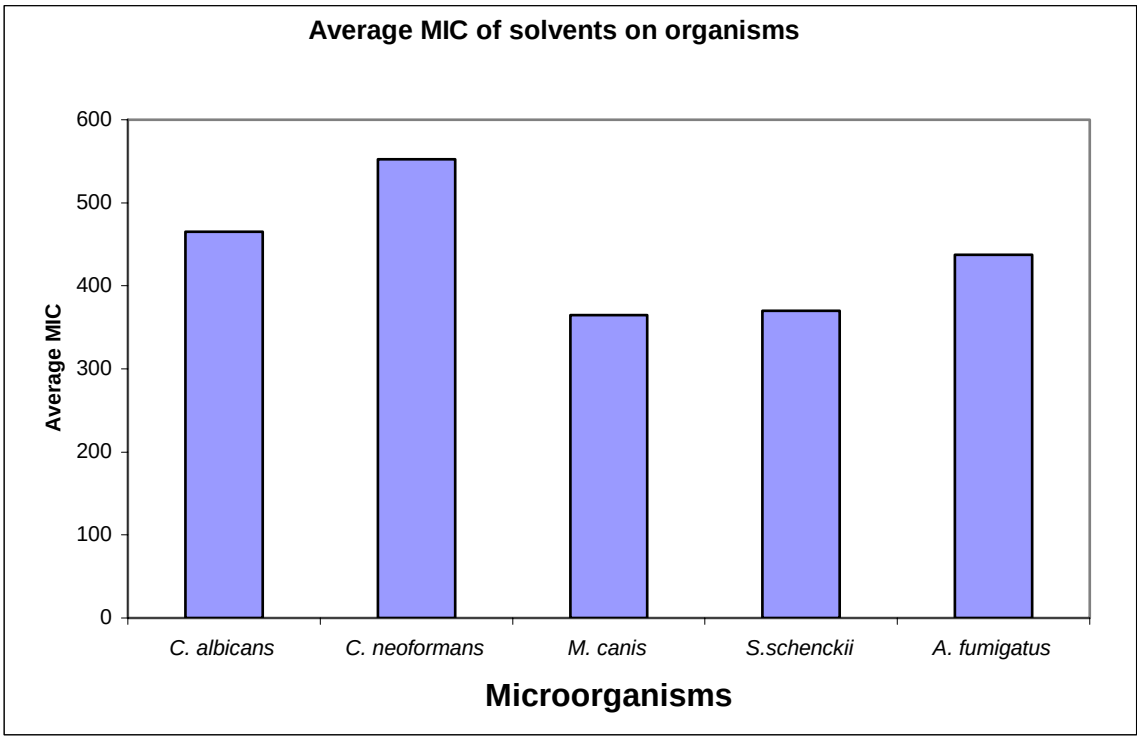


Figure 4.4. Average MIC of solvents on tested fungi

4.4. Discussion

Screening, isolation, and identification of novel compounds depends on the solubility and charge properties of the extractant. Microbial strains able to tolerate and survive in the presence of toxic organic solvent concentrations were underdeveloped until the last 5 years (Ojala *et al*., 2000). This was because of the difficulties of maintaining cell viability on highly toxic organic solvent environment and as a result of the anthropomorphic view of microbial life (e.g. aqueous media, 37 °C, pH 7.0). Also, organic solvent-tolerant microorganism, and viable cells for enzyme production in extreme environments, such as organic solvents, have received little attention, but is now growing up as a new area of extremophiles.

The solvent tolerance of the microorganisms was tested using the following solvents; DMSO, acetone, methanol and ethanol. In order to determine the maximum concentration at which different solvents would allow the test microorganisms to reach normal growth, different concentrations from 10 to 100% were used. Uninhibited growth was evaluated as no toxic effects of the solvent. Methanol and ethanol were found to be toxic to tested fungi with average MIC's of 304 and 320 mg/ml respectively as expected, based on previous studies on bacteria by Eloff (1998b). DMSO and acetone appear to be good solvents to use for bioassays, but acetone

was used in bioassays because of reasons stated earlier. The major difficulty with DMSO is the boiling point (189 ^{o}C), which is very high, fortunately it is relatively volatile and can be removed under high vacuum. If further work has to be done on extracts and it is fatty soluble in acetone that is the solvent of choice, if not DMSO may be useful.

Surprisingly, *C. neoformans, C. albicans* and *A. fumigatus* managed to survive higher concentrations of methanol, which was found to be toxic in previous studies (Heipieper *et al*., 1991). However, it was toxic to *M. canis* and *S. schenckii*. The two yeasts, *C. albicans* and *C. neoformans* were very resistant with average MIC's of 465 and 553 mg/ml respectively.

Solvent toxicity was explained considering the lipid-rich cellular membrane as the main organic solvent target by the Hansch parameter or log *P*, which is defined as the logarithm of the partition coefficient of solvent in octanol–water phase system. Organic solvents with log *P* between approximately 1 and 5 are considered extremely toxic for microorganisms. Nevertheless, the limits of solvent toxicity to the cells apparently are not strict and depend not only on strains and species assayed, but also of experimental conditions (e.g. medium, pH, temperature, ionic strength, inoculums) (Ojala *et al*., 2000).

Carlson *et al* (1991) demonstrated clearly that increasing concentrations of 6 alcohols inhibit fungal pathogens (as carried out by *Saccharomyces cerevisiae*); a correlation with increased partition coefficients into a hydrophobic milieu was also evident. This would tend to suggest that the action of these alcohols is primarily located at a hydrophobic site, possibly at the membrane.

It is well known that modest concentrations of ethanol and other alcohols lead to reduced fermentation and growth rates of organisms, which produce them, and that high concentrations are cytotoxic. While much research has been carried out (Lovitt *et al*, 1988), the methods by which these organic solvents affect the cell are poorly documented; in many cases they are simply cited as being multi- target or non-specific in their action. It is however generally agreed that the cell membrane is one of, if not "the", primary target for organic solvents, as we have seen with the differences in yeasts and moulds in our experiments.

DMSO was the least toxic of the solvents used with an average MIC of 616 mg/ml (56%) followed by acetone 512 mg/ml (64%), methanol 320 mg/ml (40%) and ethanol 304 mg/ml (38%). The danger of using ethanol or methanol is evident from the inhibition by 20% ethanol or methanol of *M. canis* and *S. schenckii*. In general the two moulds appeared to be most

resistant. Acetone was the only extractant that could be used with safety at a 50% concentration.

4.5. Conclusion

There was a variable susceptibility of the fungi to the solvents with C. neoformans was not resistant and S. schenckii was most susceptible. In spite of this it was found that DMSO and secondarily acetone can be used in fungal bioassays at higher concentrations than ethanol and methanol. Thus I recommend that where possible the use of ethanol and methanol be avoided in these tests.