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THE EFFECTS OF MIRAZID FRACTIONS ON THE VIABILITY OF SCHISTOSOMULA OF *SCHISTOSOMA MANSONI*: AN *IN VITRO* STUDY

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Abstract

Schistosma infection is an endemic disease in many developing countries including Egypt. Despite the effort to control and treat the infection with praziquantel, the development of resistance is a major health problem. Myrrh extract (Mirazid®) has been used as an alternative with conflicting results. In this study the effects of Mirazid® fractions on schistosomules of *S. mansoni* have been tested. Using column chromatography we have fractionated the molecule to 10 fractions. Two of these fractions were more potent and cause damage to the schistosomules detected by toluidine blue dyes and confirmed by electron microscopic examination. In conclusion, identification and purification of the active molecules in these fractions may potentiate the antischistosomal effects of Mirazid®.

Key words: Mirazid, Fractions, *Schistosoma mansoni,* Schistosomula SEM.

Introduction

Schistosomiasis is a chronic parasitic disease of worldwide distribution particularly those in the developing countries including Egypt (Grimes *et al*, 2015). It is estimated that at least 258 million people required preventive treatment. Schistosomiasis imposes a substantial public health and economic burden, despite the continuous control efforts (WHO, 2016). The pathological features of schistosome infections start up with the development of inflammatory granuloma formation around the deposited eggs in different tissues of the hosts with subsequent development of fibrosis and complications (Fallon, 2000).

Currentlym praziquantel is drug of choice used either in the treatment or control programs of infected populations. That is because of its effectiveness against all schistosome species, it is easy to administer, it is safe with few and transient side effects and most importantly with little economic burden due to its affordable coast (Hagan *et al*, 2004). The development of praziquantel resistance (Fallon *et al*, 1995; Ismail *et al*, 1996), and the sub-optimal efficacy against immature worms during recent infections were major limitations legitimizing the seek for more effective antischisotosomal drugs (Wu *et al*, 2011).

Mirazid® (MZD), a natural extract of myrrh tree plant is recently approved for the treatment of human schistosomiasis and fascioliasis in Egypt. Myrrh and MZD safety and efficacy had been proved by preclinical and clinical studies (Sheir *et al*, 2001; Massoud *et al*, 2004). The antischistosomal action of myrrh against *S. mansoni*, *S. haematobium* or mixed infection was first described by Sheir and colleagues; as myrrh had cure rates of up 98% (Sheir *et al*, 2001). El Baz *et al*, 2003; Abo-Madyan *et al*, 2004) had shown similar result with oral MZD. Myrrh proved to have strong antischistosomal properties, *in vitro*, due to its ability to induce the somatic muscle contractions and to cause tegumental damage of the parasite in the form of tegumental disruption, oedema and loss of the spines covering the tubercles (Hassan *et al*, 2003). Although, *in vitro*, MZD showed more damaging effects than praziquantel against adult *S. mansoni* worms and schistosomules, it did not show any significant effects in the murine model (Sharaf, 2016).

However, a multicentre study for treatment of *S. mansoni* in mice indicated that myrrh is not effective against adult schistosome (Botros *et al*, 2004). Its efficacy against *S. haematobium* in experimental animals was also limited with poor response in the form

of 22% increase in dead ova in tissues with a slight reduction (18.3%) in the number of stool eggs (Ramzy *et al*, 2010). Also, MZD had minimal cure rates of individuals infected with *S. mansoni* (Barakat *et al*, 2005).

The present study fractionated MZD and investigated the effect of MZD fractionations on schistosomules of *S. mansoni*.

Materials and methods

MZD was fractionated by silica gel column chromatography. Silica gel column chromatography was set using slurry of silica gel (mesh 70 - 230) dissolved in hexane as a stationary phase. 500mg of MZD dissolved in 4ml of dichloromethane and the solution was gently added above the surface of silica. The Pinch clamp was released to allow MZD solution to diffuse into the column. 10ml of eluent (dichloromethane in hexane) were then added to the top of the column and different MZD fractions were collected.

The elute was incubated in the continuous fume hood allowing dichloromethane to be evaporated. The fractions were dissolved in 2ml of DMSO.

Table 1 concentrations of dichloromethane and hexane in eluent used to reveal different fractions of MZD

Fractions	Dichloromethane	Hexane
1	10%	90%
2	20%	80%
3	30%	70%
4	40%	60%
5	50%	50%
6	60%	40%
7	70%	30%
8	80%	20%
9	90%	10%
10	100%	0%

Scanning Electron Microscopy (SEM): Schistosomula of *S. mansoni*, in the similar condition as before, were incubated at 37^{o}C and 5% CO_2 for eight hours. The parasites were fixed in 2.5% glutaraldehyde buffered with 0.1M phosphate for l h; washed 3 times with 0.1M phosphate buffer rinse (containing 2% sucrose). The samples were then fixed in 1% osmium tetroxide for an hour, washed 3 times with distilled water, fixed in 0.5% aqueous uranyl acetate in the dark, for 1 hour and washed with distilled water. The samples were then dehydrated in ascending grades of ethanol, 30, 50, 70, 90 and 100% and finally in dried absolute alcohol for 10 minutes each time. After critical point drying with liquid CO_2, the specimens were mounted on metal stubs with double-sided copper tape. They were coated with gold and examined by using a Phillips SEM 500 electron microscope.

The method of mechanical transformation of schistosomula was a modification of Colley and Wikel (1974). Briefly, *S. mansoni* cercariae freshly shed were transferred into a plastic universal. The cercariae were then aspirated and dispensed 10 times using a 10ml syringe with 21-gauge needle to shear off their tails. The schistosomula were washed twice in RPMI-1640 and left for 2 hours at 37^{o}C to complete their transformation before use.

The viability of schistosomula was assessed by toluidine blue exclusion test (Dessein *et al*, 1983).

One hundred schistosomula were incubated in 1ml of medium containing 2, 10 and 20µl/well of either MZD or MZD fractions for 8 hour in 24 well plates. 100 schistosomula were incubated in media containing 10µl of DMSO were used as controls.

Results

The results in table (1 & 2) and figure (1)

Table 2 Effect of MZD and MZD fractions on schistosomula of *S. mansoni* by toluidine blue dye exclusion test.

concentration	2 µl			10 µl			20 µl		
Fractions	Total	Damaged	%	Total	Damaged	%	Total	Damaged	%
Control	81	2	2.5	74	3	4	68	2	3
MZD	75	4	5	64	43	67	71	64	90
1	78	2	2.5	68	2	3	79	1	1
2	63	4	6	75	74	99	91	91	100
3	66	3	4.5	58	3	5	64	8	12.5
4	72	4	5.5	74	20	27	82	24	29
5	89	4	5	64	53	83	69	68	98.5
6	70	1	1.5	77	5	6.5	74	4	5
7	91	3	3	67	4	6	87	3	3
8	95	2	2	66	3	4.5	66	3	4.5
9	83	1	1	59	3	5	89	4	4.5
10	93	2	2	90	2	2	61	1	1.5

Full MZD molecule, fractions 2 (20% dichloromethane 80% hexane) and 5 (50% dichloromethane 50% hexane) showed significant damage to schistosomula ($p<0.05$) at concentrations of 10µl/ml and 20µl/ml compared to the control untreated group. At a concentration of 10µl/ml, fractions 2 and 5 showed significant damage compared to the full MZD molecule. Fraction 4 (20% dichloromethane 80% hexane) showed less damage at both concentrations. Other fraction showed no significant effect.

Scanning electron microscopy: The Effect of MZD full molecule and MZD fractions 2 and 5 were confirmed by SEM. Both MZD and MZD fractions showed similar pattern of damage to the surface of schistosomula in the form of surface blebs and contractions of the somules with fraction 2 showed more extensive surface damage represented by increased number of surface blebs in the same surface area.

Discussion

Human schistosomiasis is one of the most important parasitic diseases constituting a major health problem in many areas in the world including sub-Saharan Africa and Egypt. Praziquantel is now the drug of choice for treatment of all the schistosome species infecting humans and has been used extensively in schistosomes control programs in many areas worldwide (Magnussen, 2003; Hagan *et al*, 2004). MZD is an extract of myrrh obtained from different species of the *Commiphora* plant. Myrrh is a very complex mixture of compounds that varies in composition among *Commiphora* species (El Ashry *et al*, 2003).

In the literature there are controversies regarding the anti-schistosomal effect of MZD (Myrrh). Studies on both human and animal models had shown that myrrh and MZD are effective against *S. mansoni* and *S. haematobium* (Sheir *et al*, 2001; Massoud *et al*, 2004; El Baz *et al*, 2003; Abo-Madyan *et al*, 2004a; Hamed and Hetta, 2005). On the other hand, others showed that it had no effect or even minimal efficacy against *S. mansoni*, *S. haematobium* and *S. japonicum* (Botros *et al*, 2004; Ramzy *et al*, 2010; Osman *et al*, 2010; El-Malky *et al*, 2013; Lotfy *et al*, 2013).

The reason for the controversy about the efficacy of MZD is not fully excavated. Inconsistency of MZD material when obtained from natural origin is claimed to be one of the reason the drug has variable efficacy; nevertheless the inconsistency of its component varies from batch to batch (Gajbhiye *et al*, 2010; Ramzy *et al*, 2010). Another possibility is that one or more of myrrh extract constituents might have antischistosome effects while others do not and may even have deleterious effects on the hosts. If these active components could be identified and tested separately the efficacy of MZD could be predicted and may be enhanced (Sharaf, 2016).

In the present study, MZD was fractionated to 10 parts and tested the effects of each

fraction on schistosomula of *S. mansoni*. Using toluidine blue dye exclusion test, MZD fractions 2 and 5 showed significant damage of schistosomula. The severity of the damage obtained from these fractions was greater than MZD full molecule at the same concentration suggesting that purity and consentient of the compound play a vital role in the response. It was noticed that some fractions with reduced potency particularly fraction 4 and others with no effect. The damage was confirmed by SEM with fraction 2 and 5 resulted in more surface blebs and contractions of the somules.

The fractionation effect and the change in the drug potency have been shown in many compounds. For instance, fractionation of the crude ethanolic extract of the stem bark of *Tamarindus indica* showed that some fractions were more potent than the crude extract when tested against gram negative and gram positive bacterial. While the crude extract were active against 57.1% of the gram negative and 80 % of the gram positive bacterial strains, fraction 1 showed 100% activity against gram negative and 60% activity against gram positive strains. Fraction 2, on the other hand, showed 71.4% activity against gram negative and 100% activity against gram positive strains (Nwodo *et al*, 2010). Similarly, the crude ethanolic extract of Harshringar leaves showed only modest potency against *P. falciparum*; however its fractionations revealed fractions with more potent antimalarial activity (Kumari *et al*, 2012).

On the other hand, The origins of myrrh and frankincense are traced to the Arabian Peninsula. According to Herodotus (5th century BC): "Arabia is the only country which produces frankincense, myrrh, cassia, and cinnamon, the trees bearing the frankincense are guarded by winged serpents of small size and various colors." Diodorus Siculus wrote, in the second half of the first century BC, that "all of Arabia exudes a most delicate fragrance; even the seamen passing by Arabia can smell the strong fragrance that gives health and vigor." He also mentioned gold mines so pure that no smelting was necessary. The Magi, carrying myrrh, frankincense, and gold, came from the East: Arabia. The frankincense trade route, with transport by donkeys and later by camel caravans, reached Jerusalem and Egypt from the Dhofar Region of what is today Oman, through Yemen, turning north to follow the Red Sea Coast. It is likely that the same or similar species of the resin-bearing plants grew across the Red Sea in the area that is now Somalia and Ethiopia, while the collection of the gum resins was initiated in Arabia. Myrrh contributed much in the human welfare (Tonkal and Morsy, 2008).

Nomicos (2007) in USA reported that since the antiquity, genus *Commiphora* is composed of more than 200 species, and exploited as a natural drug to treat pain, skin infections, inflammatory conditions, diarrhea, and periodontal diseases. He added that in more recent history, products derived from *C. myrrha* and various other species of *Commiphora* are becoming recognized to possess significant antiseptic, anesthetic, and antitumor properties. The traditional practice and evidence-based research have supported that these properties are directly attributable to terpenoids (especially furanoses-quiterpenes), active compounds present in myrrh essential oil. Weeks and Simpson (2007) in USA presented the molecular phylogeny of *Commiphora,* a pre-dominantly tropical African, arid-adapted tree genus to test the monophyly of its taxonomic sections and to identify clades to help direct future study of this species-rich and geographically widespread taxon. Multiple fossil calibrations of *Commiphora* phylogeny proved that it is sister to Vietnamese *Bursera tonkinensis* and that its crown group radiation corresponds with the Miocene onset. Auffray (2007) in France studied the impact of the two types of antioxidant on se-bum squalene peroxidation by UV irradiation. The first type was free radical scavenger (Butyl hydroxyl toluene and an olive extract rich in hydroxytyro-

sol). The second type was the essential oil of *C. myrrha*, a singlet oxygen quencher. These properties were confirmed using the 2, 2-diphenyl-1-picrylhydrazyl test for the anti-radical capacity and 1,3-diphenylisobenzo-furan test for the capacity to quench singlet oxygen. Also, the author extended an ex vivo method to classify the efficacy of cosmetics to protect squalene by collecting sebum in vivo and irradiating it in a controlled way. The squalene monohydroperoxide formation was monitored by high performance liquid chromatography. He concluded that essential oil of *C. myrrha* gave the best protection against squalene peroxidation, and that squalene peroxidation during solar exposure was mainly because of singlet oxygen and not due to free radical attack, and that sun care cosmetics should make use not only of free radical scavengers but also of singlet oxygen quenchers.

The myrrh was approved by (FAD) US Food and Drug Administration (Ford *et al,* 1992). Mirazid was reported in several clinical and experimental trials to be a safe and effective natural herbal drug. Evident antitrematode activity was demonstrated in human fascioliasis *gigantica* (Massoud *et al,* 2001; Abo-Madyan *et al,* 2004b), and in edible animal fascioliasis (Haridy *et al,* 2003) as well as in Saudi Arabian edible animal (Abo-Zinadah, 2005) in experimental and human heterophyidiasis heterophyes (Fathy *et al,* 2005; Massoud *et al,* 2007), dicrocoeliasis (Massoud *et al,* 2003) and farm animals infected with *Fasciola* sp., *Dicrocoeleum dendriticum* and *Paramphistomum* sp. (Haridy *et al,* 2006) and as anticestode in monisziasis *expansa* (El-Shazly *et al,* 2004) and *Bertiella studeri* (Al-Mathal *et al,* 2010) and as the anti-nematode in the strongyloidiasis *stercoralis* (Massoud *et al,* 2006).

Mirazid antiprotozoa activity was proved in zoonotic *Cryptosporidium parvum* (Massoud *et al,* 2008; Abouel-Nour *et al,* 2015; 2016), in hepatic coccidiosis due to *Eimeria stidae* in rabbits (Baghadadi and Al-Mathal, 2010) and also against *Trichomonas vaginalis* in infection resistant to metronidasol (El-Sherbiny and El Sherbiny, 2011). Also, the mirazid in experimentally *Giardia lamblia* infection in Albino rats caused complete cure rate (Fathy, 2011). Moreover, Omar *et al.* (2005) in Egypt evaluated and compared hepato-toxic, genotoxic and carcinogenic effects of Praziquantel (PZQ) and Mira-zid (MZ) on adult male albino rats by assessment of serum levels of ALT, AST & bilirubin, histopathological study of the liver and cytogenetic study of bone marrow cells. 100 rats were divided into 4 groups: I- negative control, II- control rats received distilled water, III- received weekly single oral dose of PZQ (1500 mg/kg) for 6 weeks, and IV- received daily oral dose of MZ (500 mg/kg) for 6 weeks. At the end of the study 10 rats of each group were examined for the levels of AST, ALT, & Bilirubin. After scarification, liver sections were exam-ined by light microscopy. Another 10 rats of each group were submit-ted to the cytogenetic examination. PZQ induced a significant incre-ase in the mean values of AST, ALT & bilirubin with areas of hyaline degeneration, fatty changes, dysplasia & necrosis in liver sections. It induced a significant increase in incidence of chromosomal aberrat-ions as polyploidy, fragment, deletion & ring chromosome as compared with control group. MZ induced a non-significant increase in mean values of AST, ALT & bilirubin, with a normal hepatic tissue, and a non-significant increase in the incidence of chromosomal aberrations, as compared with control. On comparison of both drugs, PZQ induced a significant hepatotoxic, genotoxic and carcinogenic effects. They concluded that praziquantel was considered to be a hepatotoxic, genotoxic and carcinogenic drug. But, Mirazid® was a safe and promising anti-parasitic drug, without any hepatotoxic, genotoxic and carcinogenic effects.

Conclusion

On the light of the outcome data, further studies would be required to identify and investigate molecules with the antischisto-

some potentials of different MZD fractions. Identification of such molecules would increase the antihelminthic efficacy of MZD. It is also worth mentioning that the chemical formula of MZD was not fully recognized and marketing the drug, in Egypt, as an anti schitosomal drug would require further validation to be considered as a first choice drug in treating human schistosomiasis as well as other parasites.

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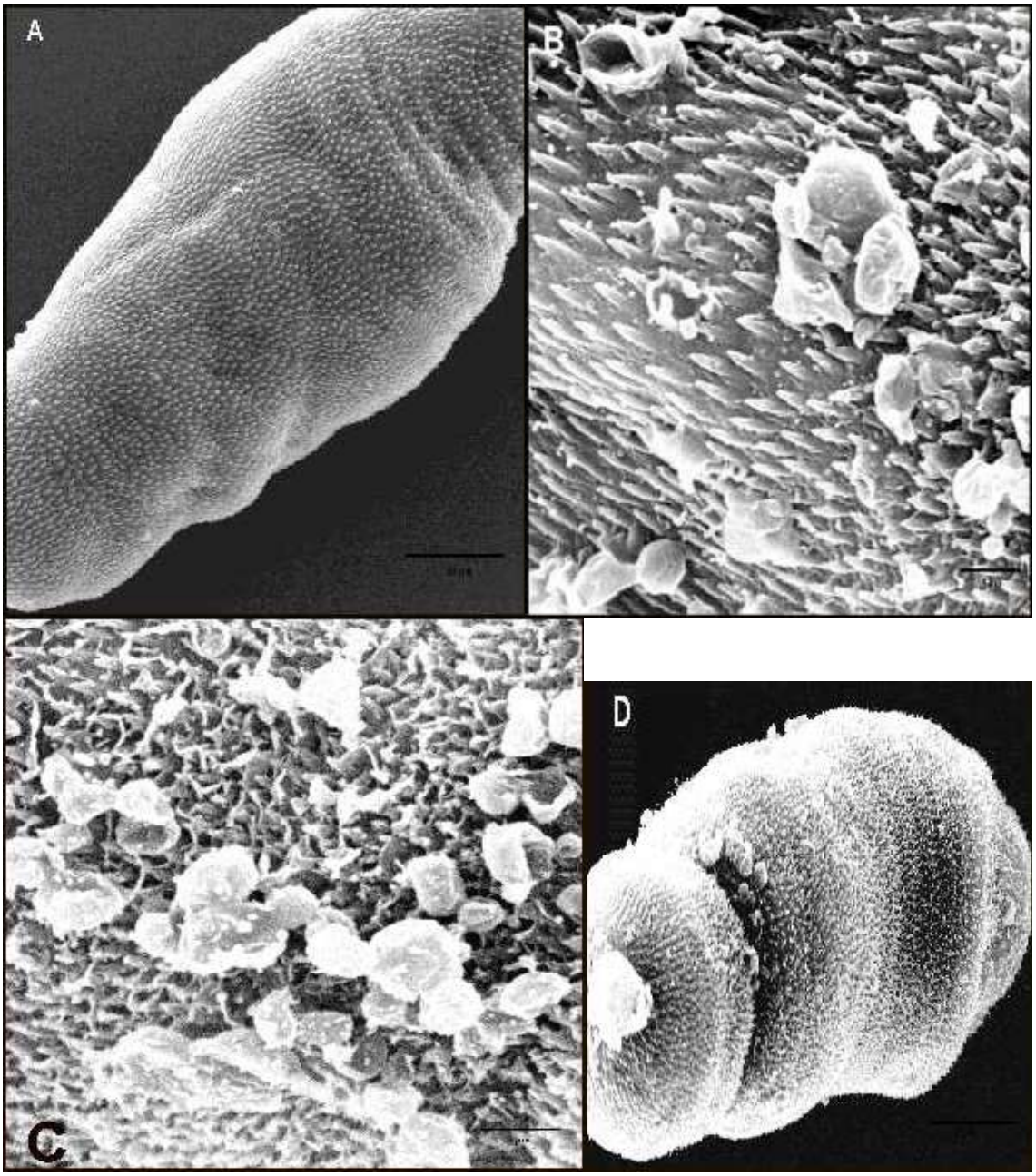

Fig.1: SEM of schistosomula of *S. mansoni*. A- Control, B- Schistosomula treated with MZD, C- Schistosomula treated with MZD fraction 2 and D- Schistosomula treated with MZD fraction 5.