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THERAPEUTIC EFFICACY OF RIFAMPICIN AGAINST *TRICHINELLA SPIRALIS* IN MICE

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Abstract

The need for new alternative treatment for trichinosis is being motivated by the growing resistance and low bioavailability of current therapies. In this study, experimental mice were used to assess the therapeutic effects of rifampicin alone or in combination with albendazole against *Trichinella spiralis*. One hundred male mice were classified into five groups of 20 mice each, G1: negative or normal control (non-infected untreated), G2: positive control (infected untreated), G3: drug control (infected and albendazole treated), G4: infected and rifampicin treated, and G5: infected and treated by albendazole and rifampicin combination. Half of the mice were sacrificed on the 10th day post infection (dpi) for the intestinal phase and the other half were sacrificed on the 40th dpi for the muscular phase. The treatment effectiveness was evaluated by parasitological, histological, and biochemical tests in contrast with positive control. Mice given albendazole and rifampicin combination gave a highly significant decrease in *T. spiralis* intestinal adult count, larval count in muscle and lowered liver activity enzymes. This was documented by the histopathology of liver, muscles and intestines.

Keywords: *Trichinella spiralis*, rifampicin, histopathology, AST, ALT, LDH, CPK.

Introduction

Trichinellosis was reported in more than 55 countries worldwide (Troiano and Nante, 2019). It is transmitted via feeding on pig meat and other infected animals (Tang *et al*, 2015), with clinical annual cases estimated to be 10,000, and about 0.2% mortality rate (Caron *et al*, 2020). Human trichinellosis is more or less rare, from religious practices and food habits, but sylvatic one was prevalent in the Eastern Mediterranean Countries including Egypt (Morsy *et al,* 2022). Even, domestic trichinellosis was detected among 1,025 rodents trapped from and around Alexandria abattoirs with a rate of 13.3% (Loutfy *et al,* 1999).

Trichinellosis minor infections are typically asymptomatic, but heavy infections, which signify the acute intestinal phase, showed signs of gastrointestinal disturbances (Sun *et al*, 2015).The most typical symptoms include pyrexia, myalgia, edema of the eyelids and face, and eosinophilia. Myocarditis, encephalitis, and thromboembolic illness might occasionally complicate the cases mainly in the elderly or impaired immune persons (Bruschi *et al,* 2019). Conjunctivitis, numbness and muscle weakness were chronic trichinellosis (Diaz *et al*, 2020).

Medications for trichinellosis are anthelmintics, mostly consist of Mebendazole®, Albendazole® and Levamisole®, but didn't effectively eliminate larvae (Chai *et al*, 2021). Mebendazole is more used, but with little impact on encapsulated larvae (García *et al*, 2014), and neither allowed for children nor pregnant females (Patziarka *et al,* 2014).

Rifampicin is one of a macrocyclic antibiotics made by *Streptomyces mediterranei*, with marked action on many gram-positive, & gram-negative organisms (Ma *et al*, 2021), and prevent bacteria producing DNA-dependent RNA polymerase (Tsankov and Grozdev, 2011). Also, it has anti-inflammatory and immunomodulatory effects (Hafeland *et al*, 2022). Ma *et al.* (2021) recommended its impact on *T. spiralis*.

This study aimed to evaluate the effectiveness of the Rifampicin® alone or combined with Albendazole® in treating *Trichinella spiralis* in experimentally infected male Albino mice.

Materials and Methods

The practical part of this study was conducted in Biology Department, Theodor Bilharz Research Institute, from February to the end of May 2022.

Experimental Design: One hundred Swiss Albino male mice, 18~25gm weight, parasite-free, were purchased from Theodor Bilharz Research Institute's Animal House, Giza. *Trichinella spiralis* infective larvae were obtained from the Cairo Governmental Slaughter House

Ethical consideration: The study protocol was approval code with Ms 16/11/2021 by Benha University's Research Ethical Committee, Faculty of Medicine, and agreed with the Institutional Regulations and Guidelines of Helsinki (2008)

Mice were starved for 12 hours prior to infection, and then 250 *T. spiralis* larvae were carefully introduced into their stom-achs (Hassan *et al*, 2019).

Drugs: 1-Albendazole 50mg/kg was purchased from Pharma Cure Pharmaceutical Co., as a suspension of 200mg/5ml. 2- Rifampicin, trade name RIFAM 100mg/5ml (60 ml suspension) was purchased from PHARCO Company (5 mg/kg).

Mice were classified into 5 main groups: G1 & G2, each was subdivided into 2 subgroups: subgroup A was sacrificed at 10^{th} day post infection (dpi) & subgroup B was sacrificed at 40^{th} dpi. G1 (20 mice), neither infected nor treated mice negative control, G2 (20 mice), infected untreated mice (positive control), G3 (20 mice), subdivided to two subgroups: A (10 mice): infected treated with albendazole on 3^{rd} dip for three days, each one got 50mg/ kg/day orally gavage, and subgroup B (10 mice): infected mice received albendazole 50mg/ kg/day started on 30^{th} dpi, and continued for 3 successive days. G4 (20 mice): Infected and treated with rifampicin 5 mg/kg orally gavage. Subgroup A (10 mice): treatment started on $t3^{rd}$ dpi, for 7 successive days (Specht *et al*, 2008), and subgroup B (10 mice): treatment started on 30^{th} dpi, for 7 successive days. G5 (20 mice): Infected treated with combined albendazole and rifampicin as the previous doses and sequences as in G3 & G4.

Detection of intestinal phase: Mice were sacrificed on 10^{th} dip for detection of intestinal adults' drug effects.

Parasitological examination: Adults were isolated and counted (Denham and Martinez, 1970). Mice were dissected for intestine, chopped into small pieces, and put in a Petri-dish of normal solution. Adults were allowed to pass from intestines to be free in saline solution by incubating the Petri-dish at 37°C for 4 hours. After shaken, the saline solution was rinsed and intestinal parts were discarded. The recovered fluids were placed in sterile tubes and centrifugated for 5 minutes at 1500rpm, supernatant was thrown away and sediment was saved for adults' count (Basyoni and El-Sabaa, 2013).

Histopathological examination: Mice intestinal samples were fixed, dehydrated, cleared, and processed for paraffin blocks, stained with hematoxylin and eosin, and examined for histopathological alterations in the intestines (Shalaby *et al*, 2010).

Muscular phase & drug effects: Experimented with mice was sacrificed and muscles were examined parasitologically and histopathologically. Muscle larvae was counted (Dunn and Wright, 1985), separated, 1% pepsin HCl and 200ml distilled water for the muscle digestion. Mixture was incubated at 37°C for 60 minutes with continuously moving by a metalic stirrer, and larvae were collected by sedimentation, and were cleaned in distilled water.

Histopathological examination: Parts of the mouse's thigh muscles were examined (Balaha *et al,* 2020), for *T. spiralis* larvae detection in muscles, and the inflammation degree in the skeletal muscles.

Biochemical examination: Blood samples were taken from experimented with mice for sera to detect ALT using alanine transaminase activity (Colorimetric, ab105134) as assay kit, also Aspartate Aminotransferase (ab105135) as an assay kit for AST activity, LDH activity by lactate dehydrogenase assay kit (Colorimetric, ab102526) and CPK by the creatine kinase activity as assay kit (Colorimetric, ab155901, Abcam, UK).

Statistical analysis: Data were collected, computerised, and analyzed by IBM SPSS software package, version 20.0. Qualitative data were described as number and percent. The significance was done at the 5% level. Statistical significance of the variation between more than two ordinal variables was done by the Kruskal-Wallis test.

Results

Table 1: Comparison between *T. spiralis* adult count in intestine among groups

Mice groups	Mean	Efficacy%	P value[a]	P value[b]
G 1 (normal negative control	0 ± 0.0	--	< 0.001	0.528
G 2 (infected positive control)	88.0± 11.17	--	--	0.001
G 3 (albendazole treated)	0.33± 0.52	87.67	0.001	--
G 4 (rifampicin treated)	59.83± 16.24	28.17	0.309	0.197
G 5 (albendazole+ rifampicin)	0.17 ± 0.41	87.8	< 0.001	0.752

P≤0.0=5 significant

Table 2: Comparison between *T. spiralis* larvae count in muscles among groups

Mice groups	Mean ±SD	Efficacy%	P value[a]	P value[b]
G 1 (normal negative control)	0 ±0.0	--	--	--
G 2 (infected positive control)	99.67±8.04	--	--	--
G 3 (albendazole treated)	27.33±2.80	72.3	0.014	--
G 4 (rifampicin treated)	42.17±4.26	57.5	0.220	0.220
G5 (albendazole+ rifampicin)	13.20±3.03	86.5	0.001	0.284

P≤0.05 =significant.

Table 3: Comparison among groups as regard ALT, AST, LDH & CPK activity in chronic stage at 40th d.p.i

Mice	ALT (U/L)		AST (U/L)		LDH (U/L)		CPK(U/L)	
	Mean ±SD	P value	Mean	P value	Mean	P value	Mean	P value
G 1	29.49±3.93	<0.001	40.61±3.2	<0.001	457±0.28	<0.001	63±0.04	<0.001
G 2	64.49±11.75	--	72.06±8.34	--	1348±0.42	--	200±0.11	--
G 3	40.08±7.38	<0.001	55.46±57.75	0.380	758±62.35	<0.001	110±0.33	<0.001
G 4	72.75±9.09	0.039	81.27±14.4	0.097	1069.8±28	<0.001	170±27.6	0.003
G 5	47.75±6.09	<0.001	62.06±10.14	0.027	868±42.35	<0.001	138±0.73	<0.001

P ≤0.05 significant, Data of G1, 3, 4 & 5 compared with G2

Parasitological data showed that mean the worm count for the positive control was 88.0±11.17. Mean number of worms in mice intestine treated with albendazole (G3), rifampicin (G4) and combined albendazole & rifampicin (G5) were 0.33±0.52, 59.83± 16.24, & 0.17±0.41 respectively. There was a high significant decrease in worm count in albendazole treated mice and combined albendazole and rifampicin, as compared to G2, with efficacy of 87.67%, & 87.8% respectively. But, no significant difference was in worm count in rifampicin treated mice when compared with (G2). Also, no significant difference was in worm count between albendazole treated mice as compared to combined albendazole and rifampicin treated mice.

Mean larvae count for positive control group was 99.67±8.04. Mean number of muscular larvae count treated with albendazole, rifampicin and combined albendazole & rifampicin were 27.33±2.80, 42.17 ±4.26 & 13.20±3.03 respectively. There was a significant decrease in larvae count in albendazole treated mice as compared to G2, with 72.3% efficacy. There was a high significant decrease in combined albendazole and rifampicin treated mice in larvae count as compared to positive control, with efficacy of 86.5%. But, no significant difference was in larvae count in rifampicin treated mice as compared to G2, and no significant difference was in larvae count between albendazole treated mice as compared to combined albendazole and rifampicin treatment.

Biochemically, rifampicin treated mice significantly reduced ALT, LDH, and CPK without significant decrease of AST in chronic infected stage as compared positive control. But, combination of albendazole and rifampicin showed significant reduced AST, ALT, LDH, & CPK as compared to positive control.

Histopathologically, G5 small intestines showed minimal inflammatory infiltration in intestinal sections, muscle sections and liver sections as compared to positive control, which showed significant inflammatory infiltration mostly affecting the villi's core and extended into submucosa, goblet cells hyperplasia, declined in ratio of crypt depth to villous height and extensive ulceration of the mucosa, and increased larvae number surrounded by severe inflammatory reaction in muscle sections and severe hemorrhage and inflammatory infiltration in liver sections of positive control. Details were given in tables (1, 2 & 3), and figures (1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16 & 17).

Discussion

Albendazole, a broad-spectrum anthelmintic medication but, of less value in treating the *Trichinella spiralis* encysted larvae (Kalaiselvan *et al*, 2007). Meanwhile, the *Wolbachia*, an intracellular bacterium infecting nematodes, is among the most prevalent parasitic microorganisms affecting the reproductive parasite in the ecosystem (Taylor *et al*, 2018). Without *Wolbachia* colonization, some host species cannot propagate or even survive (Wu *et al*, 2004), as There was a strong mutualistic link between *Wolbachia* and nematode hosts' tissues (Engelstädter and Hurst, 2006). This co-operation helped them survive since *Wolbachia* is required for the nematode host's viability, growth and fertility, and in turn the nematode host provided the necessary amino acids for the *Wolbachia*'s development (Foster *et al*, 2005). Thus, the intracellular endosymbiont may be reduced after worm sterilization. The nematodal worms' ability to survive without *Wolbachia* may be a compromised (Landmann *et al*, 2011). Due to all of these characteristics, *Wolbachia* is an intriguing target for tissue the nematodal medication treatment (Taylor *et al*, 2010).

Deborah *et al.* (2015) reported that the doxycycline could deplete *Wolbachia* and finally cause the death of adult worms. Inhibition of bacterial RNA polymerases (RNAPs) and protein synthesis is the principal mechanism of action of this antibiotic (Sulaiman *et al*, 2019). However, the doxycycline proved to be highly effective, but underappreciated antimicrobial, with broad therapeutic spectrum, exceptional bioavailability and rare evidence of serious adverse events (SAEs), being cheap and the most popular tetracycline derivative currently available (Ruhe and Menon, 2007). But, its use was limited, especially in two vulnerable patients as pregnant women and young children (Meaney-Delman *et al,* 2013).

Nevertheless, doxycycline can bind to calcium in growing bones and teeth in young children, creating deposits and/or leading to dental hypoplasia and discoloration (Sulaiman *et al*, 2019). This drawback inspired researchers to look for alternative *Wolbachia* treatments. Rifampicin, azithromycin and minocycline have been evaluated for humans as anti-*Wolbachia* chemotherapy due to the potential reports in previous studies and better safety profile (Volkmann *et al,* 2003).

In the present study, intestinal worm counts were significantly lower in all treated mice groups as compared to positive control with the best (87.8%) in combined treated mice. There was a significant decrease in larvae in muscles compared to positive control up to 86.5% but, by comparing the positive control to each of the treated mice individually, there was a marked improvement in the architecture of intestines, particularly with combined treatment with more or less almost full recovery. This agreed with Ma *et al.* (2021) in Mexico, who found that rifampicin was effective against intestinal *Trichinella spiralis*. Fahmy and Diab (2021) in Egypt, who reported that the combination of albendazole and azithromycin were highly effective in treated *T. spiralis*.

Moreover, the synergistic effect between albendazole and rifampicin considerably decreased the time needed to treat the *Onchocerca volvulous*, as its macrofilaricidal effect was quick because *Wolbachia* was nearly completely eliminated following seven days course of albendazole and rifampicin treatment (Specht *et al*, 2008). Besides, the rifampicin used orally eliminated *Wolbachia* from filariae more quickly than with the doxycycline dose and rifampicin proved safe for man and were given in short courses to patients at risk of filariasis, shortening the anti-*Wolbachia* therapy to 7-14 days (Aljayyoussi *et al*, 2017).

In the present study, the muscle cell was directly damaged by *Trichinella* larvae during their invasion and migration, as well as indirectly damaged by host's inflammatory response (Bruschi and Chiumiento, 2011). The increases in alanine transaminase, aspartate transaminase, Lactate dehydrogenase and creatine phosphokinase were connected to this injury (Kociecka, 2000).

In the present study, undoubtedly, AST, ALT, LDH & CPK were active in the chronic stage and were significantly lower in mice received combined albendazole and rifampicin than they were in positive control. This agreed with La Grange and Mukaratirwa (2014) in France, they found that CPK was a more accurate indicator of muscle injury since its highest levels coincide with the entry of larvae into muscle at 35th dpi. Moreover, both Olaniyan *et al*. (2022) and Lala *et al*. (2022) reported that the ALT & AST liver enzymes in persons treated by rifampicin was within the standard reference range for normal people over age of 18years, indicating that the drug had no short-term hepatotoxic effects.

Conclusion

The outcome data showed that both albendazole and rifampicin together exclusively must be given in trichinosis infected patients. This will avoid rifampicin antibiotic resistance as a key anti-tuberculosis medicine as this combination exhibited great activity against *Trichinella spiralis* human infection without hepatotoxicity on short-term use.

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Explanation of figures

Fig. 1: *T. spiralis* adult count in intestine among groups

Fig. 2: *T. spiralis* larvae count in muscles among groups

Fig. 3: Intestinal section of normal control group of mice showed regular villous pattern (H&E X200)

Fig. 4: Skeletal muscles section of normal control group of mice showed normal pattern and arrangement of skeletal muscle bundles (H&E X200)

Fig. 5: Intestinal section of infected control mice showed distorted villous pattern in form of marked villous atrophy (grade 3) and villous expansion by inflammatory cells (++) (black arrow) with scattered adult sections within the mucosa (yellow arrows) (H&E X200)

Fig. 6: Skeletal muscles section of infected control group of mice showed many T.S. cysts associated with dense inflammatory cellular infiltration (black arrow) (H&E X200)

Fig. 7: Intestinal section of mice treated by albendazole showed mildly distorted villous pattern (mild villous atrophy with mild expansion by inflammatory cells) (black arrow) and occasional adult worm sections within mucosa (yellow arrow) (H&E X200)

Fig. 8: Skeletal muscles section of mice treated by albendazole showed degenerated *Trichinella* cysts capsule and mild to moderate(+) infiltration by macrophages ,mononuclear inflammatory cells (H&E stain, X200)

Fig. 9: Intestinal section of mice treated by rifampicin showed many *Trichinella* adult sections within the mucosa (yellow arrows) associated with mild inflammatory cellular infiltration (black arrow) (H&E stain, X200)

Fig. 10: Skeletal muscles section of mice treated by rifampicin showed many degenerated *Trichinella* cysts with severe hemorrhage (blue arrow) with moderate (++) cellular infiltration (H&E X200)

Fig. 11: Intestinal section of mice treated by combination of albendazole and rifampicin showed absence of *Trichinella* adult worm sections, regaining normal villous pattern and minimal inflammatory cellular infiltration (H&E, X200)

Fig. 12: Skeletal muscles section of mice treated by combination of albendazole and rifampicin group showed mild inflammatory cellular infiltration (H&E, X200)

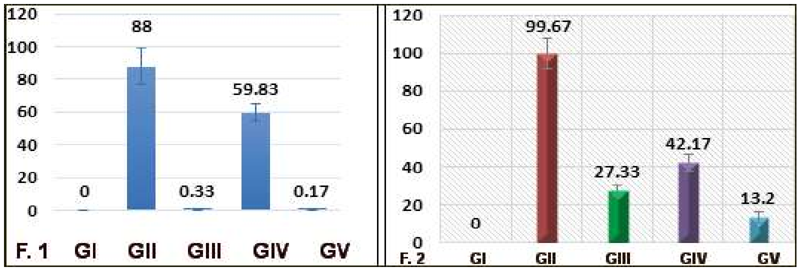
Fig. 13: Liver section of normal control mice showed regular hepatic architecture (H&E X200)

Fig. 14: Liver section of infected control showed severe inflammatory cellular infiltration (black arrow) and hemorrhage (blue arrow) with (H&E, X200)

Fig.15: Liver section of mice treated by albendazole showed moderate inflammatory cellular infiltration (black arrow) and mild (+) hemorrhage (blue arrows) with (H&E X200)

Fig. 16: Liver section of mice treated by rifampicin showed moderate inflammatory cellular infiltration (black arrow) and moderate to severe hemorrhage (blue arrow) with (H&EX200)

Fig. 17: Liver section of mice treated by combination of albendazole and rifampicin showed slight inflammatory cellular infiltration minimal hemorrhage (blue arrow) (H&E, X200)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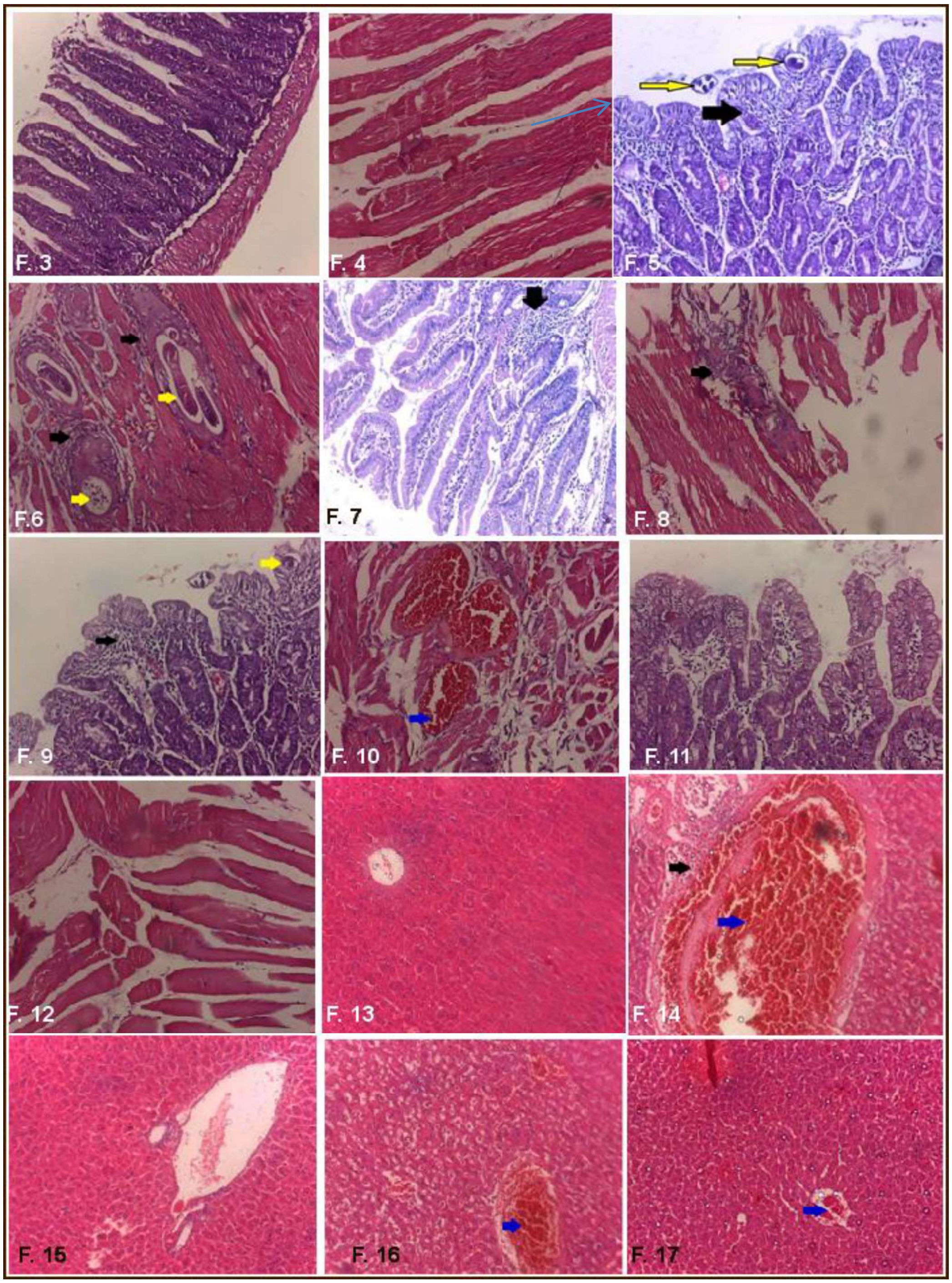
100
80
60
40
20
0
88
59.83
0
0.33
0.17
F. 1
GI
GII
GIII
GIV
GV
120
100
80
60
40
20
0
99.67
27.33
42.17
13.2
0
F. 2
GI
GII
GIII
GIV
GV


F. 3
F. 4
F. 5
F.6
F. 7
F. 8
F. 9
F. 10
F. 11
F. 12
F. 13
F. 14
F. 15
F. 16
F. 17