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Glycyrrhetinic acid and its analogs: A new class of antifilarial agents

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ABSTRACT

Although a number of chemicals have been isolated from *Glycyrrhiza glabra*, only a few have been evaluated for their biological significance. As part of our drug discovery program for antifilarial agents from Indian medicinal plants, the roots of *G. glabra* were chemically investigated, which resulted in the isolation and characterization of an antifilarial agent, glycyrrhetinic acid (GA, **1a**) effective against microfilariae (mf) in vitro (LC100: 12.5 μM; IC_{50}: 1.20 μM), but was inactive against adult worms. Further, GA (**1a**) was converted into six analogs (**2a**–**7a**) and their antifilarial potential was evaluated by studying in vitro motility and MTT reduction assays employing mf and adult worms of *Brugia malayi*. The results showed that out of six GA analogs, the benzyl amide analog (**6a**) killed adults and mf at 25 and 50 μM concentration, respectively, and inhibited 49% MTT reduction potential of the adult parasites. The IC_{50} values were found to be 8.8 and 2.2 μM for adults and mf, respectively. The SI of the compound was >60. On the other hand the octylamide analog (**7a**) required much higher concentration to adversely affect the parasites. Finally, both active amide analogs (**6a** and **7a**) were in vivo evaluated using *B. malayi*-jird model, which showed that analog **6a** possesses promising macrofilaricidal activity at 100 mg/kg, s.c. ×5 days and around 40% of the treated animals showed calcified masses of worm fragments in peritoneal cavity of the animals. To the best of our knowledge this is the first ever report on the antifilarial potential of GA analogs. Further work on optimization of the antifilarial lead is under progress.

Lymphatic filariasis (LF), a longstanding chronic disease caused by *Wuchereria bancrofti*, *Brugia malayi* and *B. timori* is transmitted through the bites of infected mosquitoes. It is prevalent in many parts of the tropics and sub-tropics of the world. Currently over 120 million people are affected by the infection with 40 million people showing chronic disease symptoms.[1] In India, more than 500 million people are exposed to infection and approximately 25 million people are known to harbor circulating microfilariae (mf) and another 19 million people suffer from filarial manifestations.[2] WHO has recognized this major health problem as one of the six important tropical diseases and launched a global programme for elimination of filariasis (GPELF).[3,4]

Diethylcarbamazine (DEC) ivermectin and albendazole are existing antifilarial drugs for human filariasis, of which DEC and ivermectin are principally microfilaricidal with limited or no action on adult parasites. DEC has been in use almost empirically for more than five decades.[5] Ivermectin mainly affects the late stages of microfilarial development while albendazole has a transient effect on early embryogenesis. Also drug resistance to ivermectin appears to be another issue of concern, especially in areas where DEC cannot be administrated. Moxidectin though looks promising in animal studies is still under development. In current therapy DEC and ivermectin are given either alone or in combination with albendazole. Advent of mass drug administration (MDA) strategy raised hope for elimination of this disease, however, unfortunately this disorder is continuing due to the technical limitations of MDA strategy.[6] This depressing perspective demands, an urgent need for new molecular structures associated with macrofilaricidal activity/or sterilizes the adult worms is, therefore, needed[7,8] since adult parasites not only produce millions of mf that are picked up by mosquito vector and transmitted, but are also responsible for the debilitating pathological lesions. Therefore, we need macrofilaricidal agents which not only adversely affect the target but should have very low or no side effect.

Since ages, several medicinal agents have been derived and developed from plants and utilized in traditional therapeutics. India has a rich tradition of using medicinal plants or their products in treating different disease conditions through Ayurveda, Unani and Siddha systems of medicine. A number of natural products with diverse chemical structures have been isolated as anticancer,[8] anti-inflammatory[9,10] and anti-diabetics[11] etc. and many of them have been modified to yield better analogs for activity. Indeed,

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several successful molecules also have emerged as drugs upon modification of the natural leads.

Over the past few years' triterpenoids from higher plants have shown a wide range of biological activities, such as antitumor,[12] antiviral,[13] anti-inflammatory[9,10,14] and anti-HIV.[15] As part of our drug discovery program on antifilarial agents from Indian medicinal plants, the literature search revealed significant antifilarial activity in pentacyclic triterpene, oleanolic acid.[16,17] This prompted us to investigate antifilarial activity in other triterpenoids found in widely used Indian medicinal plants. For this purpose, the roots of *G. glabra* were selected, which contain a unique triterpenic acid 'glycyrrhetinic acid GA (**1a**)' as a major constituent in the form of saponin 'glycyrrhizic acid'. For the isolation of GA (**1a**), the roots of *G. glabra* were extracted and fractionated according to the flow chart (Fig. 1). The TLC profile of all the fractions showed that the desired compound, glycyrrhizic acid was present as a major component in the butanol extract. Hence, the BuOH fraction was chromatographed over Silica gel H on flash. The fractions 42–58 eluted with $CHCl_3$:MeOH (85:15) afforded glycyrrhizic acid (650.0 mg), characterized on the basis of ^1H and ^{13}C NMR spectroscopic data (Fig. 2). Further, glycyrrhizic acid was acid hydrolyzed and the hydrolyzed product after work up was purified over Silica gel H on flash. The fractions 28–46 eluted with $CHCl_3$:MeOH (99:1) afforded GA (**1a**) (250.0 mg), characterized on the basis of its ^1H and ^{13}C NMR spectroscopic data.[18] (Fig. 3)

Finally, BuOH extract which contained glycyrrhizic acid as the major constituent and GA (**1a**) were evaluated for antifilarial activity against *B. malayi* using motility and MTT assays[19–21] and the results are presented in Table 1. From the results it is evident that BuOH extract was inactive against female adult worms as well as mf even at 1000 μg/ml whereas GA (**1a**) at low concn was active against mf (LC100: 12.5 μM; IC_{50}: 1.20 μM), but was inactive against adult worms even at 100 μM concentration. This prompted us to semi-synthesize some analogs of GA (**1a**) to identify better activity.

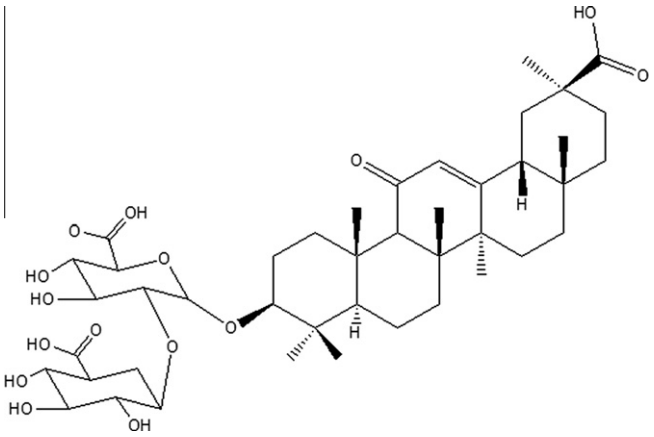


Figure 2. Characterization of pooled fraction 42–58 as Glycyrrhizic acid.

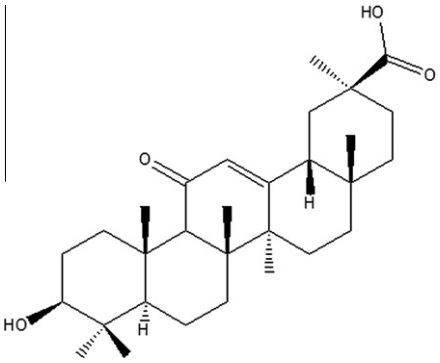
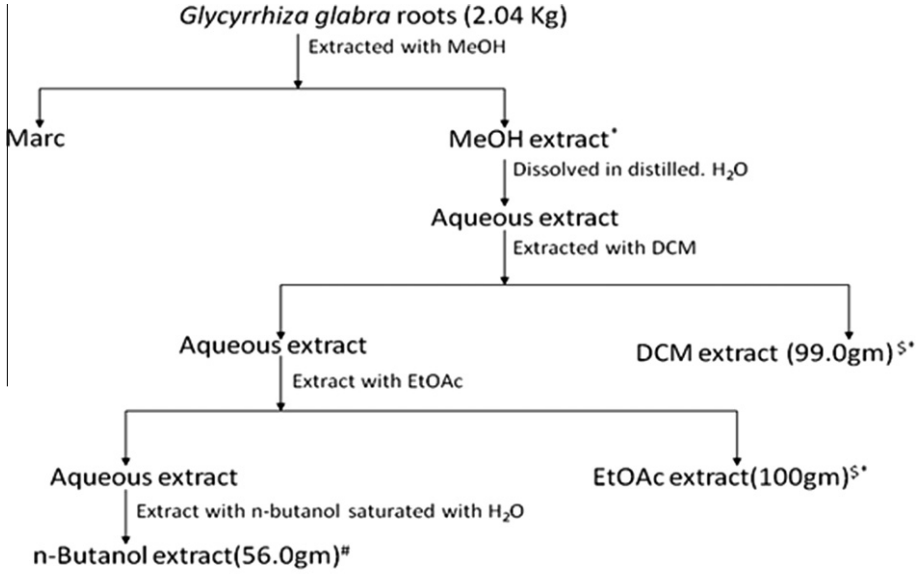


Figure 3. Characterization of pooled fraction 28–46 as Glycyrrhetinic acid.



A schematic procedure for extraction and fractionation of *Glycyrrhiza glabra* roots

$Washed with water and dried over anhydrous Na_2SO_4.
*Solvent was completely removed under vacuum on Buchi Rota Vapour.
#Solvent removed under vacuum by making azeotrop with water

Figure 1. Extractions and Isolation procedure of *Glycyrrhiza glabra*.

Table 1
Antifilarial activity of synthetic compounds tested against *Brugia malayi* adult worms and microfilariae in vitro

Antifilarial agent	Effect on female adult worm			Effect on microfilariae (Mf)		CC_{50}[a] (μg/ml)	SI	
	LC100[b] (μM) inmotility assay	IC_{50}[c] (μM) in motility assay	Mean (% inhibition) in MTT	LC100 (μM) in motility assay	IC_{50}/EC_{50} (μM) in motility assay[c]		w.r.t. adults motility	w.r.t. Mf motility
Butanol extract	>1000*	—	14.10*	>1000*	—	—	—	—
GA (**1a**)	>100	—	21.71	12.5	1.20	—	—	—
2a	>100	—	9.45	>100	—	—	—	—
3a	>100	—	10.69	>100	—	—	—	—
4a	>100	—	7.47	25	2.21	7.92	—	3.58
5a	>100	—	28.78	50	8.84	10.29	—	1.16
6a	25	8.84	48.79	50	2.21	583.58	66.02	264.06
7a	100	17.68	52.15	100	42.04	106.33	6.01	2.53
DEC-C	800	289	64	500	354	9103	31.5	25.7

SI = Selectivity Index (CC_{50}/IC_{50}); w.r.t. = with respect to.
DEC-C = diethylcarbamazine citrate.
[a] CC_{50} = Concentration at which 50% of cells were killed.
[b] LC100 = 100% inhibition in motility indicates death of parasite.
[c] IC_{50} = Concentration of the agent at which 50% inhibition in motility was achieved.
* Concn in μg/ml.

Table 2
Antifilarial activity of synthetic compounds (**6a** and **7a**) and DEC against *Brugia malayi* in jirds (*Meriones unguiculatus*)

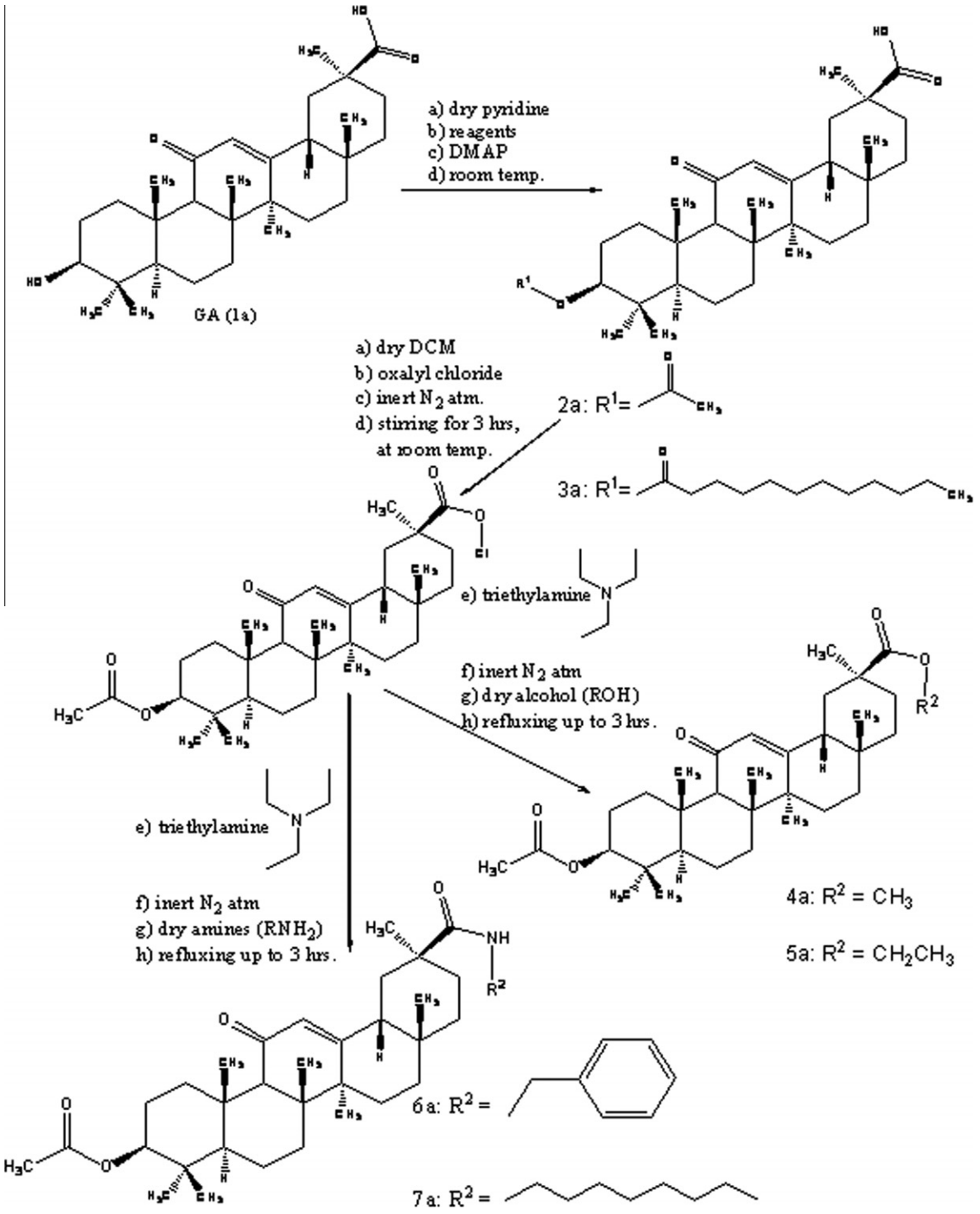
Compound no.	Dose mg/kg, s.c. ×5 days (*n*)	Status of microfilariae in peritoneal cavity on day 7/8 post initiation of treatment	No. of worms recovered (% inhibition over untreated control)	% Ster. female worms
6a	100 (5)	Highly active	5.80 ± 1.92 (54.21)***	11
7a	100 (5)	Highly active	13.33 ± 0.58	5
DEC-C	25 mg/kg (6)	Highly active	10.3 ± 2.9	11
Untreated control	Vehicle treated (4)	Highly active	12.67 ± 1.53	9

n = Number of animals; DEC-C = diethylcarbamazine citrate.
Values are Mean ± SD.
*** $P < 0.001$ (vs untreated control; student's '*t*' test).

Scheme 1 outlines the synthetic route to compounds **2a**–**7a**. For the synthesis of ester and amide derivatives in alkaline conditions required preventive protection of the hydroxyl groups in GA (**1a**), preferably with acetate. The protected 3-*O*-acetyl glycyrrhetinic acid, therefore, was obtained by reacting GA (**1a**) with acetic anhydride (2 equiv) in the presence of dry pyridine (illustrated in Scheme 1 as a representative example of the general procedure). For preparing acid chloride, the 3-*O*-acetyl glycyrrhetinic acid was reacted with oxalyl chloride (1–2 equiv) in dry dichloromethane (DCM) in a N_2 atmosphere. After 3 h of stirring, the respective dry alcohols (1.5 equiv) for esters or dry amines (1.5 equiv) for amides were added under a nitrogen atmosphere. The resulting air tight reaction mixture was refluxed for 3 h, which resulted in the formation of the desired ester and amide derivatives. Yields of derivatives are shown in the experimental part and spectroscopic data ($^1H/^{13}C$ NMR) in the Supplementary data.

The in vitro antifilarial activity of GA (**1a**) and its semi-synthetic acyl (**2a**–**3a**), ester (**4a**–**5a**) and amide (**6a**–**7a**) analogs was assessed against *B. malayi* using DEC as control. The results are summarized in Table 1. Generally, the introduction of an acyl group at C-3 (**2a**–**3a**) resulted in complete loss of antifilarial activity of GA (**1a**). On the other hand, conversion of C-30 carboxylic group of **2a** into ester analogs (**4a**–**5a**) slightly improved the antifilarial activity than those of acyl analogs (**2a**–**3a**). But when C-30 carboxylic group of **2a** was converted into amide analogs (**6a**–**7a**), the activity got decreased against mf in comparison to ester analogs, but they were found significantly active against the adult worms. This was a remarkable observation. Compound **6a** killed adults and mf at 25 and 50 μM concentration, respectively and exerted 49% inhibition in MTT reduction potential of the adult parasites as well as the IC_{50} values were found to be 8.8 and 2.2 μM against adults and mf, respectively. SI of the compound ranged between 66 and 264. Compound **7a** required 4 times higher concn (100 μM) compared to compound **6a**, to kill the adult parasites and mf; however, at the given concentration it exerted 52% inhibition in MTT reduction assay. DEC adversely affected the adult worms at 800 μM concentration and caused 64% inhibition in MTT reduction of the female parasites, whereas it inhibited mf motility to the tune of 100% at 500 μM concentration. The vehicle exposed parasites showed no inhibition in motility of adults/mf or MTT reduction of adult parasites (Table 1). The amide analog **6a** showed 32 times more activity at 25 μM and 160 times at 50 μM in adult worm and mf, respectively against the corresponding positive control, DEC whereas **7a** exhibited 16 times more activity than adults and 8 times active than mf at 100 μM.[22,23]

The amide analogs **6a** and **7a** which showed adulticidal activity were tested in vivo against *B. malayi* in jirds (*Meriones unguiculatus*) and the results are presented in Table 2. The results showed analog **6a** possesses moderate macrofilaricidal activity (54% at 100 mg/kg, s.c. ×5 days) in comparison to untreated control animals ($P < 0.001$), but was inactive against the mf in the peritoneal cavity (p.c.) of the animals on days 7/8 post initiation of treatment (p.i.t.). The percent sterile female parasite recovered was comparable to those recovered from the untreated animals. It was found interesting that 2 out of 5 treated animals showed some calcified masses of worm fragments in the p.c. of the animals indicating that the compound affected the worms.[22] In spite to this, compound **7a** found inactive against jird model. As expected the reference drug DEC-Citrate (25 mg/kg, s.c. ×5 days) was ineffective against both mf (peritoneal mf) and adult worms. Parasites recovered from untreated control animals were healthy with poor sterilization of female parasites. In summary, the compound **6a** showed moderate macrofilaricidal activity in *B. malayi*-jird model. Besides, the compound **6a** apparently did not produce any adverse effect on jirds during the 5 day-course of treatment and thereafter till the day of termination of the experiment in general behavior of the animals (Table 2).



Scheme 1. Schematic presentation of semi-synthetic acyl, ester and amide analogs (**2a**–**7a**) of GA (**1a**).

The mechanism by which these compounds exert filaricidal activity in vitro is yet unknown, but this is well documented that the plant is being used since centuries for treating asthma, bronchitis, ulcers, inflammation, allergies, tumor and fungal diseases. Besides, GA (**1a**) the plant also contains other phytochemical constituents like essential oil, chalcones, coumarins, alkaloids and flavonoids.[24] The symbiont bacteria *Wolbachia* in the filarial parasites is known to confer survival value to the parasites[25] and the elimination of *Wolbachia* by antibacterial might be a possible mechanism through which the synthetic amide analogs might be exerting antifilarial activity in vitro and in vivo. Since, DEC is known to suppress inflammation in filarial subjects[26], the other possibility would be the potential anti-inflammatory activity of GA (**1a**)[9,10,14] and probably that might have affected the adult parasites. In view of potential antifilarial activity and over all favorable pharmaceutical properties, the GA analog **6a** was identified as a lead candidate and will be further examined for detailed biological and pharmaceutical investigations. This is the first ever report of GA analog **6a** showing in vitro and in vivo adulticidal activity against experimental human filarial infection in the micromolar

range. It is thus, concluded that the discovery of the novel lead molecules might hopefully bring advancement in the safe and effective treatment of filariasis. Further, QSAR and docking guided antifilarial lead optimization is under progress, which will assist in elucidating the precise mechanism of action.

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bmcl.2013.02.115.

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