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Novel drug designing rationale against *Brugia malayi* microfilariae using herbal extracts

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ABSTRACT

Objective: To explore the effect of herbal polyphenolics on filariasis *in vitro*. **Methods:** Two herbal extracts, methanolic extracts of roots of *Vitex negundo* Linn. (Nirgundi) and leaves of *Aegle marmelos* Juss. (Beal) in different concentrations ranging from 40-80 ng/mL were tested for their antifilarial activity either alone or in combination with diethyl carbonate (DEC) (300 μg/mL) and/or H_2O_2 (0.5 mM). **Results:** Combination of DEC and each extract had significant anti-filarial effect. And fractions of both extracts were not effective as crude herbal extract. **Conclusions:** Such unique pharmacodynamics reported in this study might provide new drug development stratagem against filariasis.

1. Introduction

Novel drug designing research recommended by WHO boosts up traditional therapeutics under herbal medicinal principles which is already time tested and widely accepted across various cultural and socio-economic strata. However, the pharmacological effect of these medicines is unexplored. Our recent studies showed methanolic extracts of *Vitex negundo (V. negundo)* Linn. and *Aegle marmelos (A. marmelos)* Corr. had possible anti-filarial effect against *Brugia malayi (B. malayi)* microfilariae[1]. While the former plant reportedly contains vitexicarpin as active principle, which is a flavonoid analog with cytotoxic effect[2], however, flavonoid being from Rutaceae family is also supposed to have polyphenolics in the form of rutin or coumarin[3]. Phytochemical analysis of these plant extracts revealed the presence of polyphenolics like flavonoids in the roots of *V. negundo* Linn. and coumarins in the leaves of *A. marmelos* Corr. Apart from well documented antioxidant role, polyphenolics may also behave as pro-oxidants[4].

Interestingly, a proposed mechanism for the action of diethyl carbonate (DEC) *in vivo* involves stimulation of host innate immune response which eventually leads to elaboration of pro-inflammatory mediators with development of consequent oxidative milieu[5]. Our earlier work *in vitro* with DEC and potent oxidant H_2O_2 showed remarkable synergistic response, but failed to substantiate effective reversal through antioxidants, which prompted us to contemplate for putative apoptotic mechanism[6]. Besides direct oxidative onslaught, evidence also supports more subtle nexus between oxidative and apoptotic process[7]. Interestingly, recently *in vitro* studies have indicated apoptotic link with the action of DEC upon microfilariae[8]. Polyphenolics, particularly flavonoids are also known to induce direct apoptotic response[14].

With this perspective, we attempted to explore the possible pharmacological effect of these herbal extracts. For this purpose, above mentioned herbal extracts were tested in combination with DEC and/or H_2O_2 to detect synergistic effect. Further flavonoid enriched and depleted extracts along with whole extracts were compared against standard flavonoids to ascertain their antifilarial effect.

2. Materials and methods

The herbal extracts of each plant were prepared following standard protocol as described earlier[1]. Our earlier

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experiments showed 50% inhibitory concentration (IC_{50}) of these herbal extracts alone were around 70–80 ng/mL[1]. Hence, to find out synergism effect, downward concentration range starting from 80 ng/mL of each herbal extract combined with pre-optimized concentrations of DEC and/or H_2O_2 were selected.

Previous work with DEC alone or also H_2O_2 (until a toxic dose of 1.25 mM) did not show any effective response in vitro; however in combination, 300 μg/mL of the former and 0.7 mM of the latter were almost complete loss of motility[6]. Hence in this study, DEC (Sigma) and freshly prepared H_2O_2 were used at the concentration of 300 μg/mL and 0.5 mM, respectively, because DEC and H_2O_2 alone at these dosage were ineffective against the filarial parasites *in vitro* and even in combination showed a meager response. Also, the known flavonoids, catechin and quercetin were tested as standards (dose corresponding to the flavonoid content of the crude extract) in the study along with the flavonoid enriched (ethyl acetate fractions of the plant at a concentration corresponding to the IC_{50} of respective plant extract) and the flavonoid depleted (polyamide filtered fraction of the crude extract at a concentration corresponding to the IC_{50} of respective plant extract) fractions of the herbal extracts.

2.1. Collection and preparation of B. malayi microfilariae

Microfilariae of *B. malayi* were obtained by lavage from the peritoneal cavities of infected jirds (*Meriones unguiculatus*). Microfilariaes were collected and washed with RPMI 1640 medium (with various supplements) for in vitro experiments. The animals in the study was approved by Institutional Animal Ethics Committee and allowed Control and Supervision of Experiments on Animals norms.

2.2. Concentration of flavonoids by ethyl acetate fractionation

The flavonoids in the extracts were concentrated based on the principle of liquid-liquid extraction. Briefly, 15–20 mL double distilled water was added to 30 mL of each extract and 100 mL ethyl acetate was added to the mixture, which was then shaken vigorously for 15 min. The ethyl acetate phase from the two phases was separated and transferred into another beaker so that a semisolid residue rich in flavonoid remained as a precipitate after evaporation at room temperature[9].

Further fractionation of these ethyl acetate fractions of the plant extracts into different constituents with varying polarity was carried out by silica gel column chromatography using a gradient of solvents. Briefly, five fractions of *A. marmelos* (namely n- hexane; n- hexane: ethyl acetate= 60:40; ethyl acetate: methanol= 90:10; methanol: water= 90:10; water) and four fractions of *V. negundo* (namely dichloromethane; chloroform; methanol: water= 50:50; methanol) were obtained.

2.3. Depletion of flavonoids by polyamide fractionation

Polyamides have high affinity for phenolic hydroxyl groups of the flavonoids through their amide carbonyl functional groups which is suitable for separation of flavonoids[10], Therefore, polyamide filters were used to deplete flavonoids content of extracts. Briefly, 10 mL of crude plant extracts were passed through the polyamide membrane filters (0.2 μm).

2.4. Estimation of flavonoid content

Flavonoid content in the crude, ethyl acetate fraction and the polyphenolic depleted fraction of the herbal extracts were estimated by the method described by Marinova *et al*[11] with certain modifications. Briefly, either 1 mL the herbal extracts or the catechin standards (10–300 μg/mL) was added to 2 mL double distilled water in the test tubes. Later, 0.3 mL of 5% $NaNO_2$ was added. The tubes were placed in vortex and stayed at room temperature for 5 min. At the 6th min, 0.3 mL of 10% ethanolic $AlCl_3$ was added and stayed at room temperature for another 5 min. The volume was made up to 10 mL with 2.4 mL of double distilled water. The chromogen was estimated spectrophotometrically at 510 nm against blank. The total flavonoid content was expressed as mg catechin equivalents/100 gm of the extract.

2.5. In vitro screening for anti filarial activity against B. malayi

Approximately 100 microfilariae in 100 μL RPMI were introduced into 96-well micro-culture plates. Microfilariae of *B. malayi* were treated individually with plant extracts, extract with DEC, extract with H_2O_2 and combination of extract, DEC and H_2O_2 in the above mentioned dose range. The ethyl acetate fractions with highest flavonoid content and the flavonoid depleted fractions (at a concentration corresponding to IC_{50} doses of each of the herbal extracts) along with the standard flavonoids (at a concentration corresponding to IC_{50} doses of each of the herbal extracts) were also tested against the microfilariae in a similar manner as described[1]. Simultaneously suitable controls were run. The plates were incubated at 37 ℃, 5% CO_2 incubator for 48 hrs. After incubation the number of live and dead microfilariae in each well were counted, respectively, and percentage of motility was calculated by following formula:

$$\frac{\text{No. of live and motile microfilariae}}{\text{No. of recovered microtilariae}} \times 100$$

The percentage loss of motility was calculated from the above formula as % loss of motility = 100 – % of motility calculated. Each condition of the experiment was repeated thrice and the results were represented as mean±SEM.

3. Results

Two herbal extracts namely, roots of *V. negundo* Linn. (Nirgundi) in 70% methanol, leaves of *A. marmelos* Juss. (Beal) in 100% methanol alone and in combination with DEC (300 μg/mL) and/or H_2O_2 (0.5 mM) were checked for their antifilarial activity in three different concentrations (40, 60 and 80 ng/mL). The herbal extracts had enhanced effect on the microfilariae in combination with DEC. When the extracts were complemented with H_2O_2, complete loss of motility was achieved for *V. negundo* starting from the lowest dose. On the contrary, for *A. marmelos* a notable reduction in effect was observed at the lower dosage (*i.e.* in combination the extract and H_2O_2 mutually nullified their

individual effect). However, at higher dose, complete loss of motility was noted again. When the microfilariae were incubated with the individual herbal extracts, DEC and H_2O_2 altogether, 100% loss of motility was achieved in entire dose range (Figure 1 & 2).

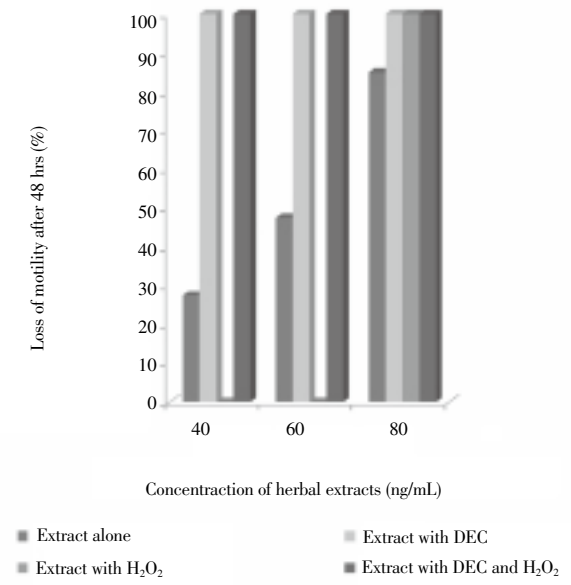

Figure 1. Comparative evaluation of effect of *A. marmelos* alone and along with combination of DEC and/or H_2O_2.

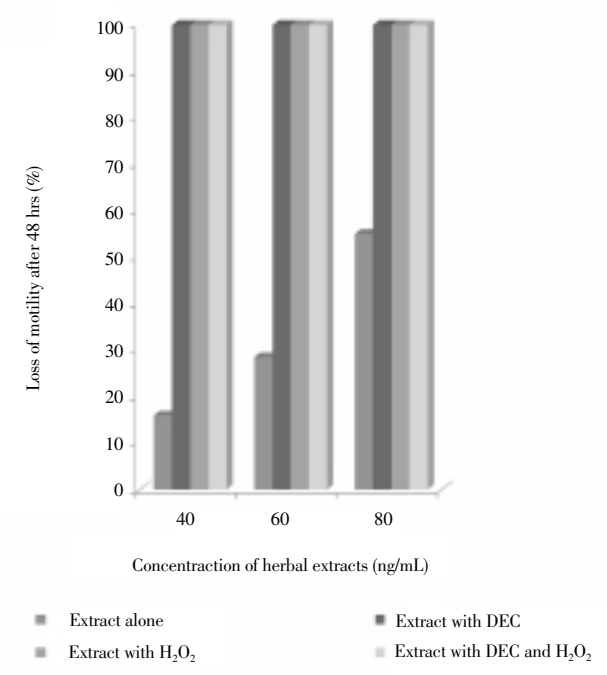

Figure 2. Comparative evaluation of effect of *V. negundo* alone and along with combination of DEC and/or H_2O_2.

The ethyl acetate extracts of the two plants were further fractionated by the column chromatography and the fraction with highest flavonoid content confirmed by spectrophotometric estimation (hydro-alcoholic fractions for *A. marmelos* and *V. negundo*) were tested for antifilarial activity. Fractions of both two plants were unable to achieve a comparable effect as produced by the crude herbal extracts on *B. malayi* microfilariae. The flavonoid depleted fractions also did not show any loss of motility. Similarly, the known standards flavonoids, catechin and quercetin produced insignificant effect on the parasites in *in vitro* conditions (Table 1).

Table 1
The flavonoid content & the corresponding pharmacological activity.

Type of extraction	Flavonoid content (mg/100 gm)	Loss of microfilarial motility (%)
A. marmelos crude extract	61.83	50.00 ± 0.61
A. marmelos flavonoid enriched fraction	105.33	14.00 ± 0.19
A. marmelos flavonoid depleted fraction	7.00	6.00 ± 0.05
V. negundo crude extract	88.83	54.00 ± 0.59
V. negundo flavonoid enriched fraction	97.67	11.00 ± 0.14
V. negundo flavonoid depleted fraction (polyamide fraction)	3.00	5.00 ± 0.71
Catechin 1	75.33*	9.00 ± 0.08
Quercetin 2	75.33*	8.00 ± 0. 09
Control 1	–	3.00 ± 0.45
Control 2	–	4.00 ± 0.64

* indicates the standard flavonoid concentration calculated from the mean of the flavonoid content at the IC_{50} doses of the *A. marmelos* crude extract and *V. negundo* crude extract.

4. Discussion

In the present study, two herbal extracts (roots of *V. negundo* and leaves of *A. marmelos*), both of which individually showed confirmed anti-filarial effect in our earlier study[1], were combined individually with DEC and/or H_2O_2. With DEC both of these extracts separately showed uniform synergism at a significant level. Whereas, when combined with potent oxidant H_2O_2, vitex root extract but not the latter showed remarkable synergistic impact over the entire dose range. Extracts of bael leaves in combination with H_2O_2 showed paradoxical protective effect at lower doses, whereas at higher doses, it turned to be filaricidal. Our earlier work had shown that the active ingredients of these herbal extracts contained various polyphenolic compounds namely coumarin and flavonoids in bael and nirgundi, respectively[1]. Since it is quite well documented that polyphenolic agents might show concentration-dependent role reversal from apparent anti-oxidant to pro-oxidant[4], therefore the observed flip-flop response with the combination of bael extract and H_2O_2 was regarded to be the result of probable dose-dependant conversion of redox potential of the active herbal ingredients. Based on this oxidative hypothesis, it might be speculated that

at lower concentrations, the polyphenolics (Coumarins) in *A. marmelos* possibly mounted an apparent resistance against the oxidative stress induced by H_2O_2. However, at higher concentration of the extract, the polyphenolic level seemed to be sufficient to turn pro-oxidative for acting in tandem with the oxidative prowess of H_2O_2 as evidenced by augmentation in the pharmacological response.

Conversely, DEC has no known oxidative effect as H_2O_2. In combination with DEC (otherwise devoid of any *in vitro* effect), both of these extracts individually showed 100% loss of motility of the worms at the concentration range, even lower than their respective IC_{50} levels. This result reflected that there was an overall synergism between the herbal extracts and DEC, which could not be explained in obvious oxidative terms. Moreover, uniform synergistic effect with the combination of *V. negundo* extracts and H_2O_2 also did not appear to follow any redox flip-flop. In our earlier study, we had reported significant synergism between DEC and H_2O_2, which could not be effectively reversed by conventional antioxidants[6]. Reported evidences suggested that filarial parasite was actually quite resistant to direct oxidative assault owing to its unique protective armament[12]. From these evidences the feasibility of the apparent oxidative rationale is still questionable.

In this rather perplexing juncture, it is worth mentioning that recently, evidence of apoptosis has been found in the effect of DEC *in vitro*[8,13]. Besides being potent oxidant, H_2O_2 is a well established inducer of apoptosis. These facts impel us to surmise that although oxidative mechanism might emerge superficially, however, a more profound apoptotic rationale looms larger in the overall antifilarial effect. It is confirmed by the uniform 100% loss of motility recorded in all the concentrations of any of these herbal extracts in combination with DEC (may be considered as inducer of apoptosis) and H_2O_2 (a potent oxidant as well as a known apoptosis inducer). The combined effect of these two agents is quite pervasive to shoot up a formidable apoptotic response in combination with the herbal extracts.

In context to the apoptotic hypothesis, the most pertinent query must focus on the active principle of the herbal extract which might be pro-apoptotic. As discussed earlier, apart form the usual redox property, polyphenolic (particularly flavonoids) compounds have directly show inductive effect on apoptosis[14]. In this connection, it may be relevant to note that vitex plant in this study has been reported to contain cytotoxic flavonoids like vitexin and vitexicarpin which are pro-apoptotic[15]. In this light, it is quite tempting to speculate that the observed synergism between vitex extract and H_2O_2 even at the lower dose range might be due to the presence of potent flavonoids which interacted with H_2O_2 in more pro-apoptotic terms than to take part in an oxidative nexus. Relative higher flavonoid content detected in vitex plant extracts than that of bael extract in this study might explain such synergistic impact even at lower dose of vitex extract. With DEC, a pro-apoptotic but not an oxidant, both two extracts showed remarkable synergism. This suggested direct apoptotic synergism between herbal flavonoid and DEC rather than any redox effect. Hence, the common herbal ingredient which might be implicated as pro-apoptotic appeared to be the flavonoids.

To answer the most intriguing query in this virtual clash between oxidative and apoptotic postulates, it should be kept in mind that oxidative stress might be an important precursor of apoptosis[7]. This is particularly important since the putative crucial player in the entire pharmacodynamics is the herbal flavonoids which are reportedly considered both as conditional pro-oxidative as well as pro-apoptotic agent. Hence it may not be prudent to overrule the oxidative component out rightly. Recent evidences has greatly challenged the view of oxidative stress as a uniform death signal and explained the crucial role of redox regulation in cell survival and stress induced signaling pathways owing to its dose dependent dual behavior[16]. At lower levels, oxidative signal prevent apoptosis but at higher level the same turns to be pro-apoptotic[17,18]. This is strikingly in accord with our finding, which recorded similar survival effect. At lower level herbal flavonoids as anti-oxidant tend to oppose the pro-oxidative role of H_2O_2 with a resultant lower oxidative load; whereas, at higher level both mutually promoted their individual oxidative impact. Observed lower flavonoid content in bael extract as opposed to vitex extract clearly supported possible anti-oxidant role of flavonoids that pacified the oxidative potency of H_2O_2 at lower dose with an apparent role reversal to augment the same at higher concentration.

Thus, flavonoids being considered as potential active principles in these herbal extracts might be responsible for the antifilarial impact. We attempted to validate this view by reducing the flavonoid content from the extracts using polyamide filtration. The filtered fractions showed significant reduction in the flavonoid level as opposed to the respective crude extracts. Such fractions when tested for their biological activity, showed very poor activity against the crude extracts, indicating the vital importance of such component in the pharmacological effect.

Further these plants were extracted in ethyl acetate followed by fractionation through chromatography and tested to have higher flavonoid content than crude ones. These flavonoid enriched fractions as well as the standard flavonoids (quercetin and catechin), quite surprisingly failed to achieve any better effect than those found with the crude extracts. This is in clear contradiction with the reductionist principle which is based on a single active principle mediated effect. In traditional therapeutics, however, it is quite well established that holistic formulations with the whole herbal extract have definitive therapeutic edge over a supposed single active ingredient[19, 20]. Based on the evidence, though the importance of the herbal flavonoids is established undoubtedly, it can be perceived that the effect of this agent is largely dependent on the combinatorial effect

of the other constituents present in the whole extracts.

Pending in-depth work to furnish definitive experimental proof, the results of this study underscored the importance of a hypothetical modality involving targeted oxidative and/or apoptotic rationale based on herbal flavonoids. Recently, an Indian group had already shown the antifilarial efficacy of flavonoids in filarial animal model, which standed in support of the projected drug candidate found in the study[20]. However, our study for the first time advocates utilizing flavonoid based herbal formulation as a novel drug designing stratagem against filarial parasites.

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Conflict of interest statement

We declare that we have no conflict of interest.

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