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THE COMPARATIVE EFFECTS OF PURIFIED FRACTIONS OF *Vitex negundo L.* (lagundi) AND CRUDE EXTRACTS OF *Cassia alata L.* (akapulko) AND *Artemisia vulgaris L.* (damong-maria) ON INFLAMMATORY PROCESSES. *IN VITRO*

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INTRODUCTION

The three plants of interest in this study are widely distributed throughout the country. *Vitex negundo L.* has been recommended in the treatment of cough while *Cassia alata L.* has been found to be effective in the treatment of fungal infections of the skin. On the other hand, *Artemisia vulgaris L.* is used for its anti-inflammatory effects (Quisumbing, 1978). However, their exact mechanism of action remains unclear.

The purpose of this study is to determine the immuno-modulatory effects of the purified fractions of *V. negundo* and crude extracts of *C. alata* and *A. vulgaris* on the following:

1. Human polymorphonuclear leukocyte chemotaxis movement
2. Phagocytosis by human polymorphonuclear leukocyte
3. Classical complement mediated hemolysis, and
4. Alternative complement mediated hemolysis

MATERIALS

Fresh leaves of the plants were obtained from various sites: *V. negundo* from the Bureau of Plant Industries, *C. alata* from a transplanted plant from Meycauayan, Bulacan and *A. vulgaris* from a propagated plant originally obtained from Pangasinan. The harvested leaves were washed and homogenized in a Waring blender within two hours. Distilled water was added to the leaves for homogenization in the following proportions: *V. negundo* 1:2 (w/v), *C. alata* and *A. vulgaris*, 1:3 (w/v). Following homegenization, the extracts were filtered, stored in Eppendorf tubes and frozen. These were lyophilized and kept at -20°C. Prior to use, the dry weight of the lyophilized extract is measured and dissolved in the appropriate buffer solution and reconstituted according to the intended concentration to be used for the extract.

The purified fractions of *V. negundo* were obtained from Dr. Fabian Dayrit (PIPAC/Department of Chemistry, Ateneo de Manila University). Due to the small amounts of the different fractions, only 1 mg of the purified fraction was added to 'spike' the crude *Vitex* extract solutions.

METHODS

1. Human polymorphonuclear leukocyte chemotaxis
   (Adapted from Cates, K.L. *et al.*, 1978 and Maderazao, E.G., Woronick C.L., 1978).

2. Polymorphonuclear leukocyte phagocytoxis
   (Adapted from Burrel R., Mascoli, C.C., 1970 and Carpenter, P.L., 1975).

3. Classical complement mediated hemolysis
   (Adapted from Harrison, R.A., Lachmann, P.J., 1988 and Gee, A.P., 1983).

4. Alternative complement mediated hemolysis
   (Adapted from Pangburn M.K., 1988).

RESULTS

I. Chemotaxis

Figure 1 shows the results for *A. vulgaris* and *C. alata*. Analysis of the results by one-way ANOVA show significant differences due to extract with $p = 0.000$. With multiple range tests (Tukey-HSD), it was determined that significant differences in chemotactic indices exist between *C. alata* and the control while there is no significant difference in the chemotactic indices of the control and *A. vulgaris*.

For *Vitex* fractions casticin and chrysoplenol D (see Fig. 2), one-way ANOVA shows significant difference at $p = 0.000$ in the mean indices between groups. The multiple range tests (Duncan test and Tukey-HSD) show that the difference is between the control and the two fractions; however, the two fractions do not significantly differ from each other.

From the data of means, it is apparent that *C. alata* significantly enhances chemotaxis, *V. negundo* fractions casticin and chrysoplenol D significantly inhibit chemotaxis. *A. vulgaris* appears to also have an inhibitory effect, although this did not attain a significant value.

II. Phagocytosis

Analysis of the mean values of *A. vulgaris* and *C. alata* by one-way ANOVA and multiple range tests show significant difference between the mean values of the extracts with that of the control. In addition, the effect of *C. alata* is dose-dependent. For the *Vitex* fractions (chrysoplenol D, lagundin and 2'-musaenosidic acid), one-way ANOVA and multiple range tests on the mean values also show significant differences between the control and the fractions. However, there seems to be no significant difference in the effect among the fractions themselves. Hence, *C. alata* significantly inhibits phagocytosis in all concentrations in a dose-dependent manner. *A. vulgaris* also inhibits phagocytosis in all concentrations. *Vitex* fractions chrysoplenol, lagundin and 2'-musaenosidic acid all significantly inhibit phagocytosis (see Figure 3 and Figure 4).

III. Alternative Complement Mediated Hemolysis

A one-way ANOVA for the corrected hemolysis absorbance values (CHAV) of each test substance demonstrates that *A. vulgaris*, at a dose of 1:1 significantly inhibits alternative complement mediated hemolysis (ACMH). A dose of 1:10, while also inhibitory, did not attain significance. Doses of *A. vulgaris* of 1:100 and 1:1000 were not different from that of the control.

In another test where hemolysis due to *A. vulgaris* was tested against those of *C. alata* and *Vitex* fractions iso-orientin and lagundin, at a dose of 1:100, *A. vulgaris* did not differ significantly from control values (see Figure 8).

The data of the dose response for *C. alata* also demonstrate a significant effect due to treatment, using ANOVA for split-plot design (see Figure 6).

Analysis of the CHAV for *C. alata* further demonstrates that *C. alata* significantly enhances ACMH at a dose of 1:10, as is depicted in Figure 6. While the 1:5 dose also enhances ACMH, the values did not attain significance. The analysis also shows that the 1:5 dose and 1:10 belong to the same homogenous subset.

In the subsequent run using a 1:100 dose of *C. alata* however, in a series with other plants (Figure 8), *C. alata* was also effective at this more dilute dose in significantly enhancing the CHAV due to the alternate complement pathway.

Results of the CHAV for the *Vitex* fractions indicate that agnuside significantly inhibits alternative complement mediated hemolysis when given at a dose of 1:10. While 2'-musaenosidic acid was also inhbitory at this dose, the difference from the control did not attain significance (Figure 7).

On the other hand, *Vitex* fractions iso-orientin and lagundin at a dose of 1:100 had mean CHAV values greater than that of control, but the difference was not significant (Figure 8).

The dose of the extract was reduced to 1:100 because of the paucity of the fractions. No runs could be performed for the other *Vitex* fractions (casticin and chrysoplenol-D) because these fractions

were already used up. Thus, it appears that of the *Vitex* fractions, only agnuside has a tendency to inhibit the alternative complement mediated pathway. The iso-orientin and lagundin fractions may have a tendency to enhance hemolysis, but the difference from control was not significant. No observations were done for the other *Vitex* fractions because of lack of the material. As mentioned, at this dose, *C. alata* enhanced complement activity while *A. vulgaris* has no effect.

IV. Classical Complement Mediated Hemolysis

Figure 9 depicts the corrected hemolytic absorbance values attributable to the classical complement pathway for the different plants *A. vulgaris, C. alata, Vitex* fractions iso-orientin and lagundin, and control.

Analysis of variance of the CHAV show that while the means for all of the plant extract group were lower than those of the control, and therefore appear to be inhibitory, none of the plants, when used at a dose of 1:10 could inhibit the classical complement-mediated hemolysis (CCMH) to a level significant at $p = 0.05$.

However, in a run where two other *Vitex* fractions, agnuside and 2'-musaenosidic acid, at a dose of 1:10, where compared with control, agnuside, with a mean reading of 0.1273 was significantly lower than the control value of 0.4908, indicating that *Vitex* agnuside significantly inhibits the CCMH pathway. These results are shown in Figure 10.

DISCUSSION

In the literature, extracts from plants have been shown to affect the inflammatory process. Anti-complement protein activity has been shown in some extracts (Oshima Y. *et al.,* 1988; t'Hart L.A. *et al.,* 1988; van der Nat J.M. *et al.,* 1989; Thabrew M.I. *et al.,* 1991; Kapil A. and Moza N. 1993). Effect on other inflammatory processes have also been shown in vitro: chemotaxis (Dorsch W.E. *et al.,* 1990) and phagocytosis (Thabrew M.I. *et al.,* 1991).

Phytochemical analysis done on *A. vulgaris* (Geisman T. A. and Ellestad G.A., 1962) *C. alata* (Gupta D. and Singh J., 1991) and *V.*

negundo (Dayrit F., 1989) has shown the presence of flavonoid and iridoid compounds. Flavonoids have been shown to inhibit cAMP phosphodiesterase (Nikaido T., 1989), cGMP phosphodiesterase (Ruchstuhl M. et al., 1979), lens aldolase reductase (Varma S.D. et al., 1975) and also inhibits the lipo-oxygenase and cyclo-oxygenase pathways in arachidonic acid metabolism (Moroney M.A. et al., 1988; Ferrandiz M.L. and Alcazar M.J., 1991). Flavonoid effect on neutrophil cytokinesis has also been demonstrated (Kenny M.T. et al., 1990). Flavonoids have been shown to inhibit the oxidative burst in neutrophils (Pagonis C. et al., 1986). Some flavonoids have also been identified as natural bradykinin antagonists (Calixto J.B. and Yunes R.A., 1991).

Vitex fraction iso-orientin has been shown to inhibit histamine release in rat mast cells (Dayrit F., 1989). The analgesic effect of *C. alata* has been compared with kaempfero 3-osophoroside in rats and mice (Palanichamy S. and Nagarajan S., 1990) and was shown to have comparable analgesic effects. In another species, *C. occidentalis*, an investigation on the mechanism of anti-inflammatory activity seem to point to inhibition of phospholipase A2 (Sadique J. et al., 1987). The anti-inflammatory effects of *A. vulgaris* has been demonstrated in carrrageenan-induced edema (Quisumbing, 1978) and in carragean-induced chemical conjunctivitis (Gavino B. et al., 1987).

Crude aqueous extracts of *V. negundo* have been previously performed in this laboratory using the above tests and the results are discussed in a previous paper (Caoili, Sagpao and Tigno, in preparation). In the above mentioned studies, *Vitex* was shown to have significant effect on chemotaxis, inhibiting this process significantly. The study confirms this result, and attributes the reduction in chemotaxis to fractions casticin and chrysoplenol D of *Vitex*. Unfortunately, there is very little of the purified fractions available, so that not all fractions could be included in all tests. In the previous study, in the presence of *Vitex* crude extracts, phagocytosis was not inhibited. In the present study, phagocytosis was indeed inhibited by fractions chrysoplenol D, lagundin and 2'musaenosidic acid, indicating that results using purified extracts are indeed more specific than crude aqueous solutions of *Vitex*, both classical and alternative complement-mediated hemolytic pathways were inhibited but not to a significant degree. In the present study, *Vitex* fraction agnuside was demonstrated to produce significant

reduction in both alternative and classically-mediated hemolysis at a dose of 1:10. Other fractions were either not observed or were observed at a more dilute dose of 1:100, so that no significant effects were observed. From the above data, it appears that the well-known anti-inflammatory actions of lagundi or *Vitex negundo* is partly explained by its ability to inhibit chemotaxis, phagocytosis and complement activation, both via the alternative and classical pathway. It also appears that these activities reside in different fractions of the plant extract, such that it is imperative that these fractions be isolated in order that their desired effects can be maximized. As has been mentioned, the fractions given to us for testing were of very little amount, so that no observations could be made for the other tests on some of them. Based on our results, the fractions agnuside, casticin, chrysoplenol D, lagundin and 2'-musaenosidic acid appear to have good anti-inflammatory potentials, so that it is recommended that more of these fractions be isolated. Fractions iso-orientin and lagundin appeared to have no effect on the tests of complement activation, although the dose was rather dilute. If more of these fractions could be made available, then it would be possible to re-test them in more concentrated amounts using our currently available *in vitro* assays.

Cassia alata or akapulko is well-known for its fungicidal properties. As such, it is one of the more popular herbal remedies promoted by the Department of Health, and has been the object of many awards bestowed by the PCHRD. However, much to the consternation of many of its users, it produces many undesirable side effects, primarily itching. Our current study shows that there is indeed a scientific basis to this complaint. This herb promotes leukocyte chemotaxis as well as enhances activation of the alternative complement pathway, even at a dose of 1:100. Thus, while the plant may serve as an immune-stimulant with effective anti-fungal properties, one cannot ignore its other activities. It is recommended that the plant be further chemically analyzed, and re-subjected to testing, so that the pro-inflammatory compounds can be eliminated, and the fungicidal fractions isolated so that their salutary activities can be enhanced.

Artemisia vulgaris or damong-maria significantly inhibited phagocytosis at a dose of 1:100 and inhibited the alternative pathway of complement activation at a dose of 1:1. Further testing should be done on the plant to isolate the actual ingredient so that its anti-inflammatory action can be maximized.

SUMMARY

In summary, four tests were employed to characterize inflammatory activity: phagocytosis, chemotaxis, the alternative and classically-mediated hemolytic pathways. An enhancement of any of the above processes would indicate an elevated natural immune response and therefore an activity which would promote inflammation. A suppression of any of the following processes would indicate a possible effect towards inhibiting inflammation. Based on the above parameters, the response of the different plant extracts are as follows:

EXTRACT	Chemotaxis	Phagocytosis	ACMH	CCMH
Vitex iso-orienti	no observation	no observation	no effect 1:100	no effect 1:10
Vitex lagundin	no observation	inhibit signif.	no effect 1:100	no effect 1:10
Vitex agnuside	no observation	no observation	signif. inhibit, 1:10	signif. inhibit, 1:10
Vitex 2'-mus a.	no observation	inhibit signif.	no effect, 1:10	no effect, 1:10
Vitex casticin	inhibit signif.	no observation	no observation	no observation
Vitex chrysopl	inhibit signif.	inhibit signif.	no observation	no observation
A. vulgaris	no effect	inhibit signif.	signif. inhibit, 1:10	no effect, 1:10
C. alata	enhanced signif.	inhibit signif.	enhanced, 1:10 and 1:100	no effect, 1:10

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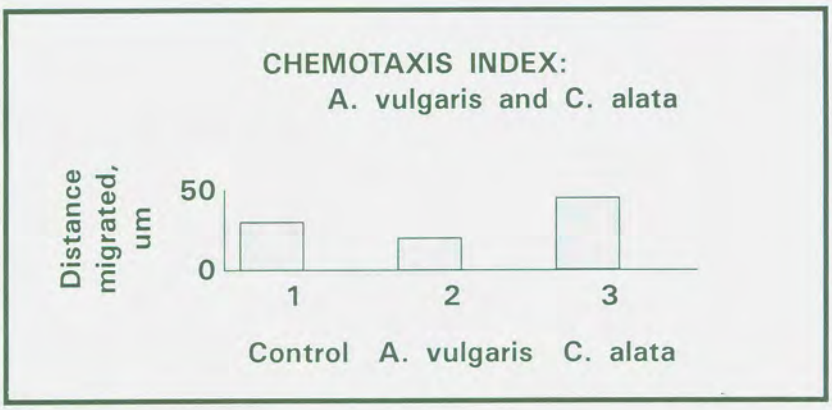
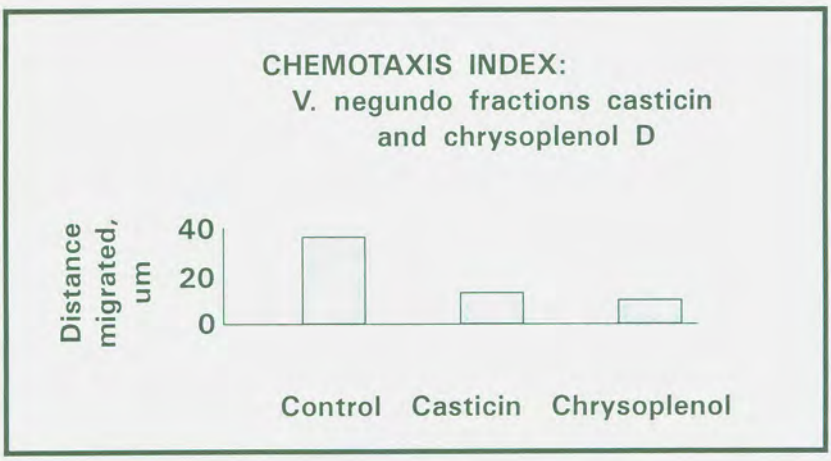


Figure 1



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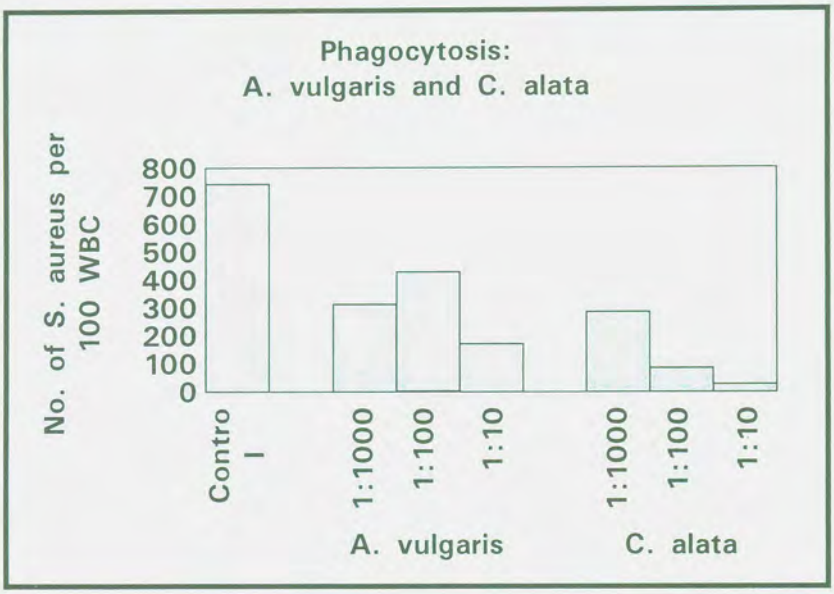

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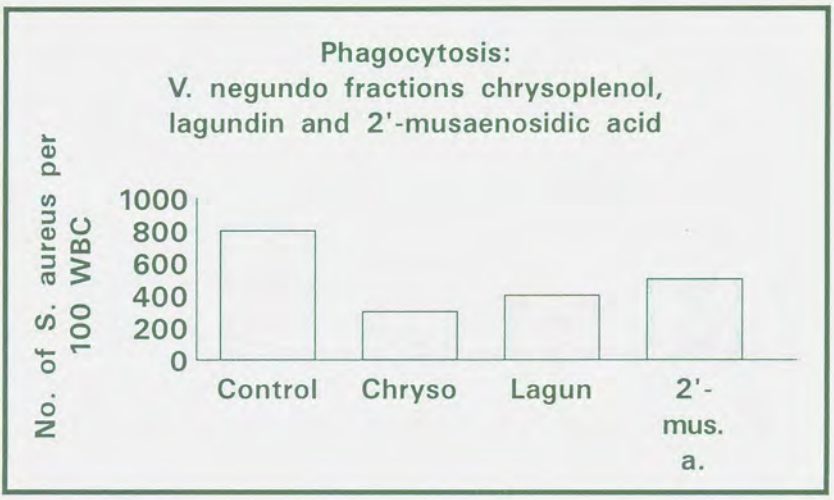

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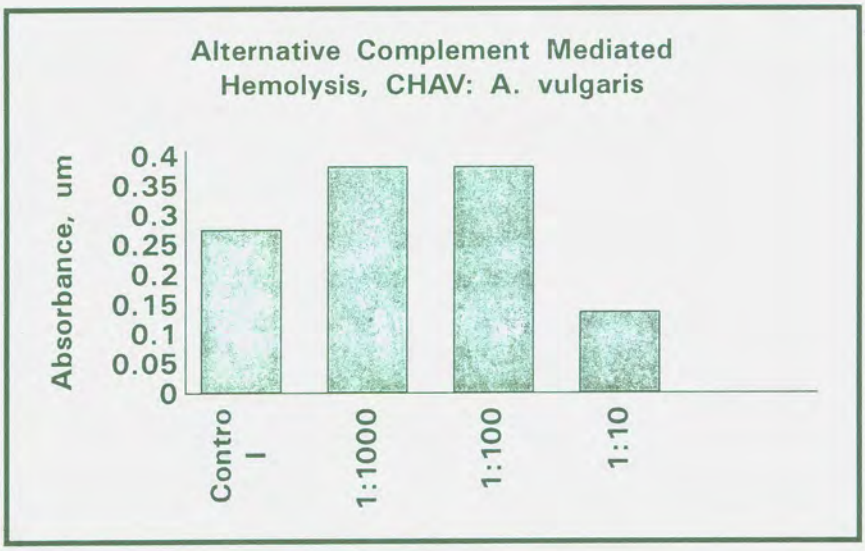

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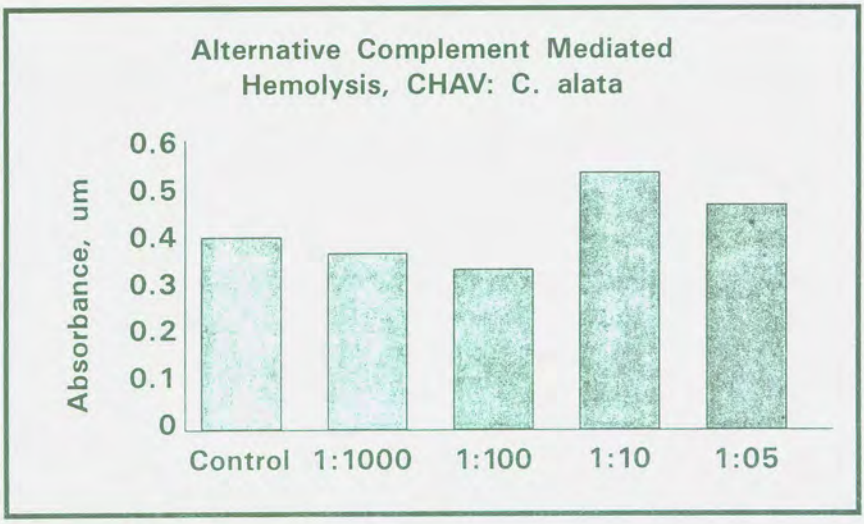

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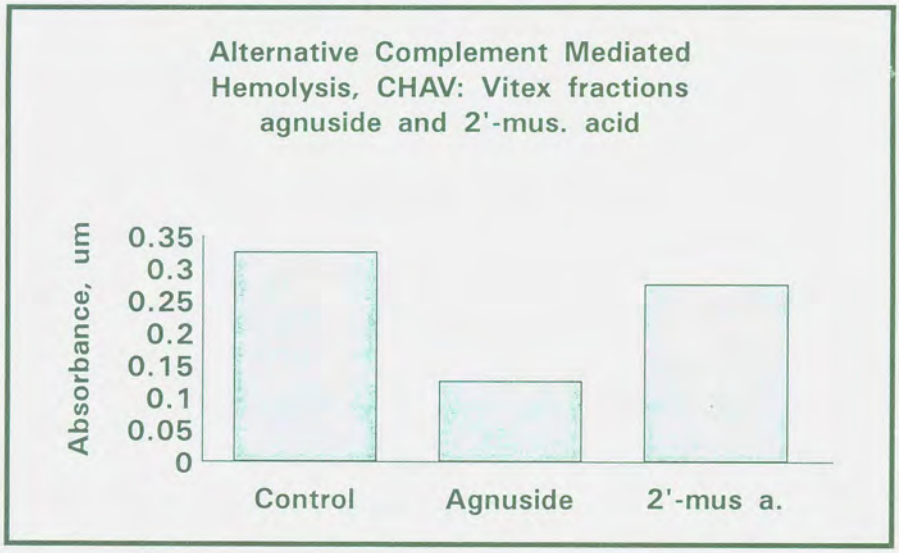

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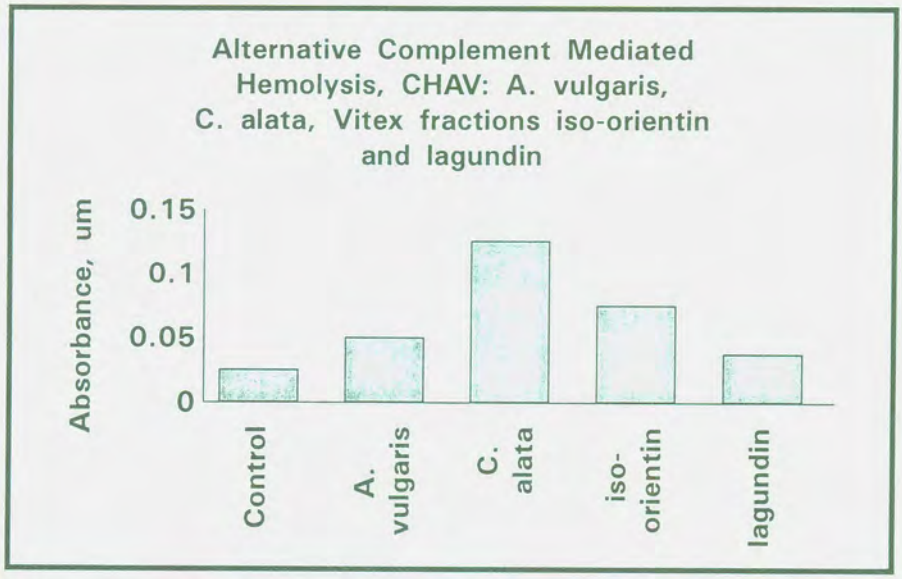

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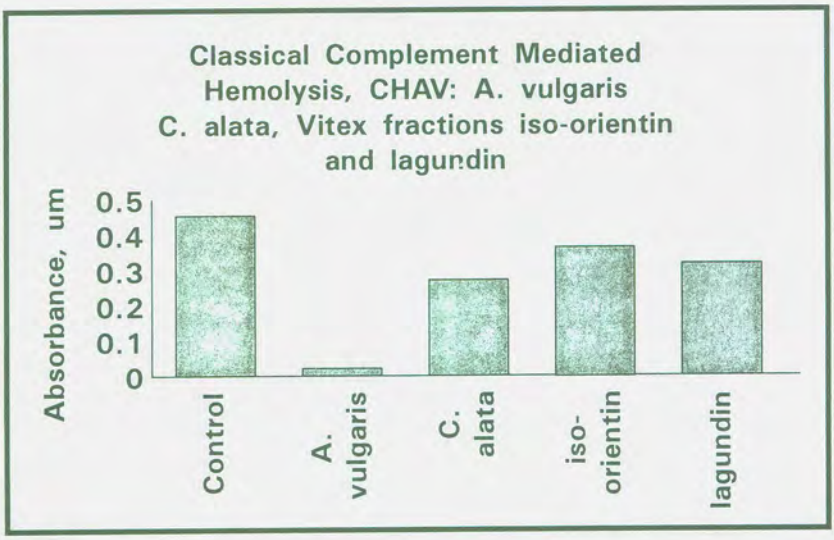

Figure 9

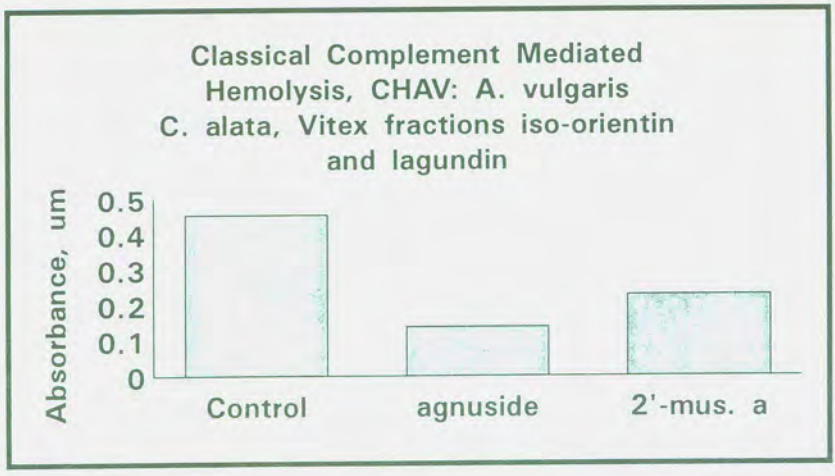

Figure 10