CHAPTER V

IN VIVO STUDIES

5.1 INTRODUCTION

Since the introduction of topical corticosteroid formulations, their use has become widespread, being prescribed for a large variety of dermatological conditions. This widespread use has created a need for a reliable method of assessing the various dosage forms with respect to potency, bioavailability and bioequivalence. Clinical trials are laborious, costly and difficult to man as well as being impractical for preliminary screening of large number of drugs or their dosage forms. A number of methods have been developed for the screening of corticosteriods and their topical formulations, the most frequently used one being the human skin blanching assay which is a non-invasive, simple, convenient, reproducible and relatively cheap technique. The basis of this method is that glucocorticoids, when applied topically on human skin, undergo percutaneous absorption resulting in apparent vasoconstriction of superficial vasculature and skin blanching(1). The most powerful vasoconstrictors are those substances which clinical studies have shown to be the most effective topical antiinflammatory agents(2).

In the present study, the skin blanching assay was used as a tool to compare the efficacy of the liposomal formulations found promising in the in vitro studies with the conventional formulations and to select a liposomal formulation for each of the drugs under study, for conducting clinical trials.

5.2 EXPERIMENTAL

5.21 Materials :

Johnsonplast adhesive tapes (Johnson and Johnson Ltd., Bombay), legend stencil with 7mm x 7mm square, disposible syringes (Rugby, USA).

5.22 Preliminary Screening :

42, healthy, male and female Caucasian volunteers between the age of 22 to 55 years who had not received topical or systemic corticosteroids for atleast 6 weeks prior to the investigation were screened for their ability to elicit the skin blanching response on application of formulation CTC. For this, a small piece of adhesive tape with a 7mm x 7mm cut square was applied to the flexor aspect of both forearms of each volunteer. 4-5 mg of the formulation was applied in the cut square and the site occluded using another piece of adhesive tape on one arm while it was kept open (unoccluded) on the other arm. After 6 hours of application, the tapes were removed gently and the forearms washed with soap and water. After one hour, the blanching was graded as excellent, average or poor. Only those volunteers who elicited an average blanching response were included in further studies.

5.23 Blanching assay methodology (3) :

Volunteers proved to be average blanchers in the preliminary screening were used in this study. Adhesive tapes

with 7mmx7mm cut squares were applied to the flexor aspect of both forearms of each volunteer (Plate 5.1) in such a way that the tape was 2.5 cms away from the elbow and the wrist. The formulations to be compared for their blanching potential were filled in 1ml disposible tuberculin syringes, the needles of which had been cut in order to facilitate the extrusion of the formulated product. The syringes were filled immediately prior to use to minimize any possible interaction between the preparation and the plastic matrix of the barrel of the syringe. The coded formulations (Table 5.2) were applied to the designated test site as per the randomization chart (Table 5.3) of the volunteer by a research worker uninvolved in the evaluation process. Around 4-5 mg of each formulation was extruded from the syringe and spread over the application site using a different glass rod for each preparation. The sites were occluded by using another adhesive tape (Plate 5.2). The formulations were allowed to remain in contact with the application sites for 6 hours, after which, the tapes were removed slowly, to reduce erythema and to prevent possible stripping of the epidermis. The sites were then gently washed with soap and water and patted dry with a towel. Blanching scores (Table 5.4) were read by a panel of three blinded observers, which included a pharmacologist and 2 research workers, after one hour of removing the tapes. The arms of the volunteers were placed horizontally on a desk, directly in front of the observer in day-light and the pallor was assessed by observing the

TABLE 5.1

SETWISE DIVISION OF FORMULATIONS AND BASES TESTED FOR THEIR SKIN BLANCHING RESPONSE.

Set	Formulations
I	KTG, TPM, T_1, T_3, T_7, T_8, T_9, T_{10}, T_{11}, T_{17}, T_{21} and HPMC K4M gel base
II	KTG, ETG, CTG, PTG, ATC, CTC, KLG, ELG, CLG, PLG, HPMC K4M gel base, HPMC E4M gel base, Carbopol gel base, PVA gel base, Aqueous Cream base, CMC base.
III	KFG, FPM, F_1, F_2, F_3, FCC, HPMC K4M gel base, CMC base.
IV	KCG, CPM, C_1, C_2, C_3, CCC, HPMC K4M gel base, CMC base.

KEY TO TABLE

KTG = TRMA in HPMC K4M gel base

TPM = TRMA physical mixture in HPMC K4M gel base

ETG = TRMA in HPMC E4M gel base

CTG = TRMA in Carbopol gel base

PTG = TRMA in PVA gel base

ATC = TRMA in Aqueous Cream base

CTC = TRMA in CMC base

KLG = Liposomal TRMA in HPMC K4M gel base

ELG = Liposomal TRMA in HPMC E4M gel base

CLG = Liposomal TRMA in Carbopol gel base

PLG = Liposomal TRMA in PVA gel base

KFG = FLU in HPMC K4M gel base

FPM = FLU physical mixture in HPMC K4M gel base

FCC = FLU in CMC base

KCG = CLO in HPMC K4M gel base

CPM = CLO physical mixture in HPMC K4M gel base

CCC = CLO in CMC base.

FIG.5.1 : FLOWSHEET FOR THE SEQUENTIAL PROCEDURE FOR THE SKIN BLANCHING ASSAY.

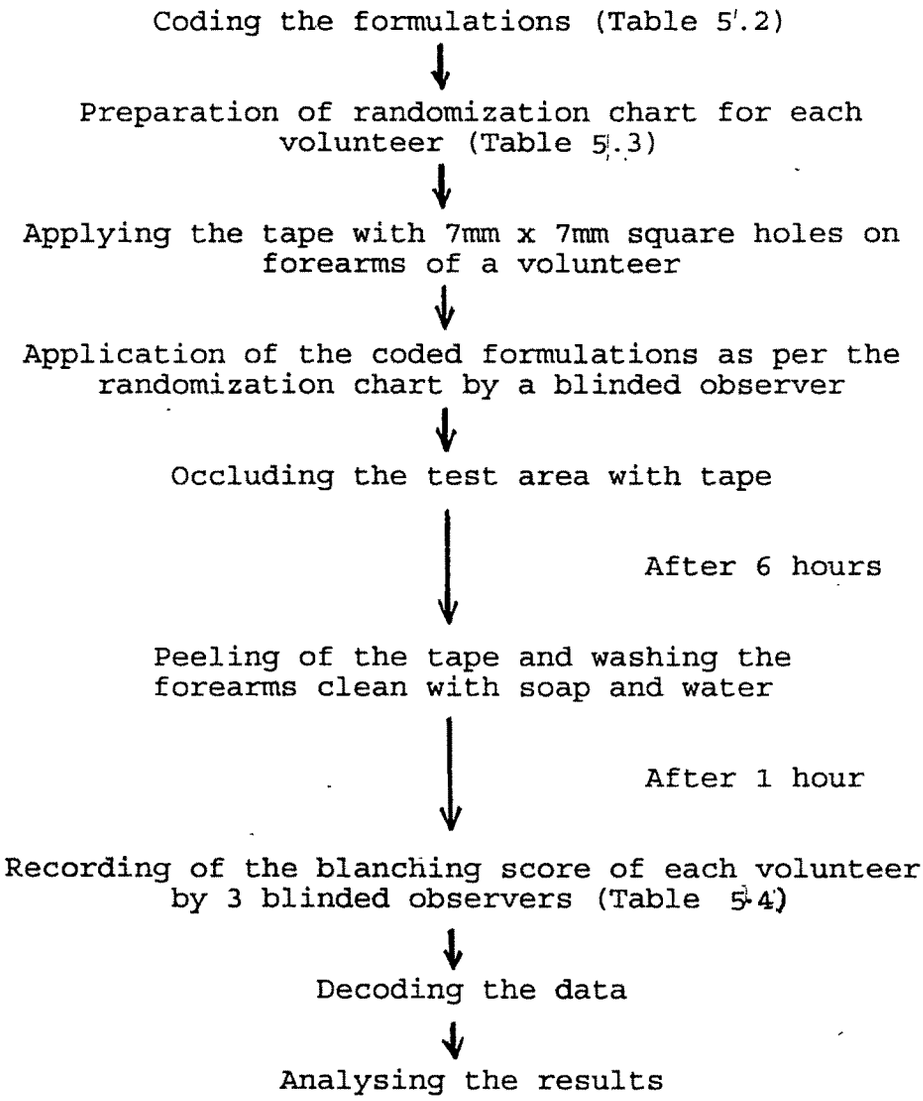


TABLE 5.2

A REPRESENTATIVE EXAMPLE OF HOW FORMULATIONS WERE CODED FOR THE SKIN BLANCHING ASSAY.

Coding for Set III and Set IV

Formulation	Code
F_1	Y_1
F_2	Y_2
F_3	Y_3
KFG	Y_4
FPM	Y_5
CFC	Y_6
HPMC K4M gel base	Y_7
C_1	X_1
C_2	X_2
C_3	X_3
KCG	X_4
CPM	X_5
CCC	X_6
CMC base	X_7

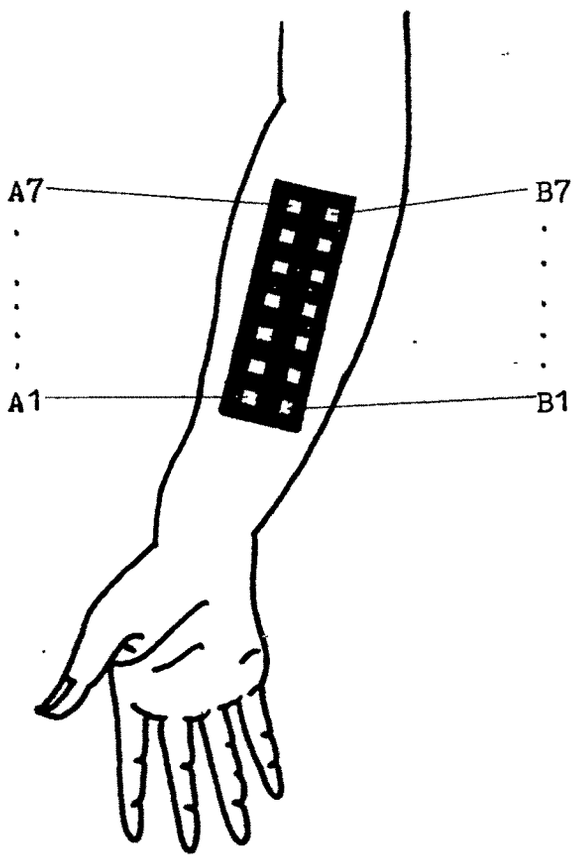


FIG. 5.2 : DIAGRAM SHOWING HOW SITES WERE ASSIGNED ON AN ARM. (Squares on the side of the thumb were designated as A1, A2... from the palm to the elbow while those towards the little finger as B1, B2....)

TABLE 5.3

A REPRESENTATIVE RANDOMIZATION CHART FOR A VOLUNTEER.

Name (No.) : A. Patel (2)

Age : 26

Sex : F

Set under test : III AND IV

Time of starting test : 9.20 a.m.

Time at which tape is to be removed : 3.20 a.m.

Time at which blanching scores are to be recorded : 4.20 a.m

Site	Formulation to be applied on Right hand	Left hand
A_1	X_3	Y_2
A_2	X_4	Y_1
A_3	X_2	Y_4
A_4	X_1	Y_3
A_5	X_6	Y_6
A_6	X_5	Y_5
A_7	X_7	Y_7
B_1	Y_3	X_3
B_2	Y_4	X_5
B_3	Y_2	X_4
B_4	Y_1	X_2
B_5	Y_6	X_1
B_6	Y_5	X_6
B_7	Y_7	X_7

Photograph of the blanching response of this volunteer to this set is shown in Plate 5.3.

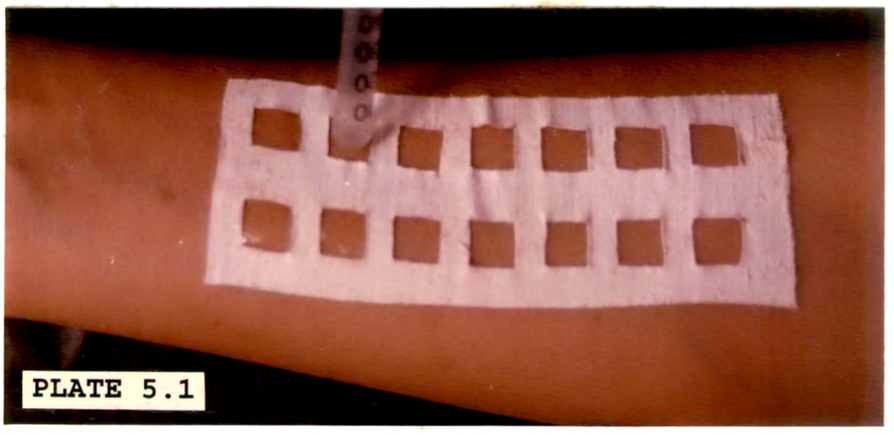


PLATE 5.1

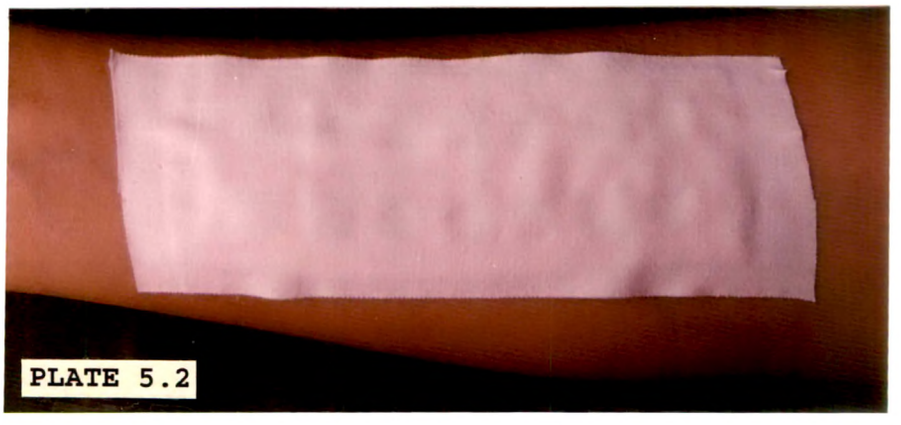


PLATE 5.2

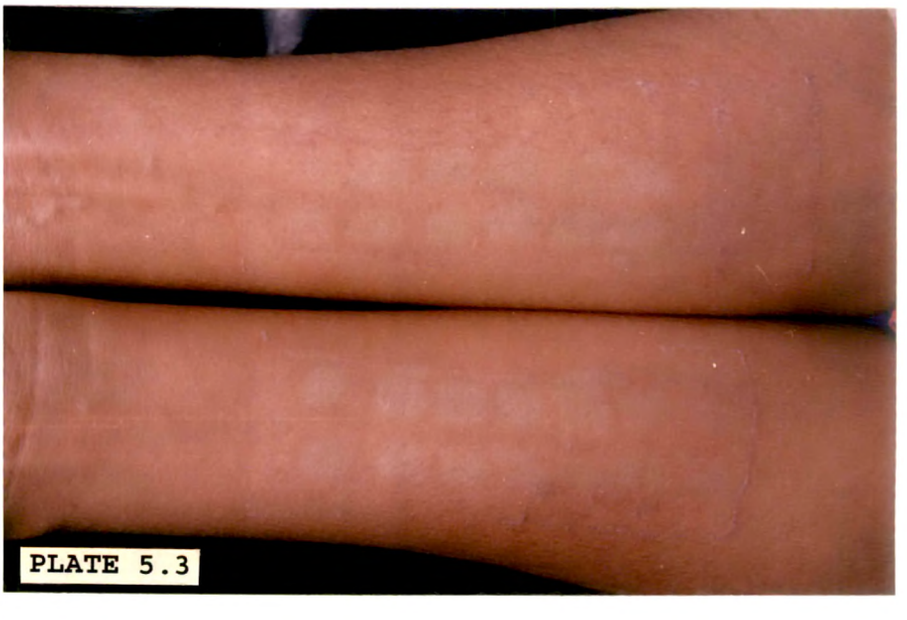


PLATE 5.3

TABLE 5.4

A REPRESENTATIVE BLANCHING SCORE CARD OF ONE OBSERVER FOR A VOLUNTEER.

Observer : 1

Volunteer (No.) : A. Patel (2)

Site	Score	
	Right hand	Left hand
A_1	3	4
A_2	4	3
A_3	4	4
A_4	4	4
A_5	2	4
A_6	2	3
A_7	0	0
B_1	3	4
B_2	4	4
B_3	4	4
B_4	4	4
B_5	2	4
B_6	2	3
B_7	0	0

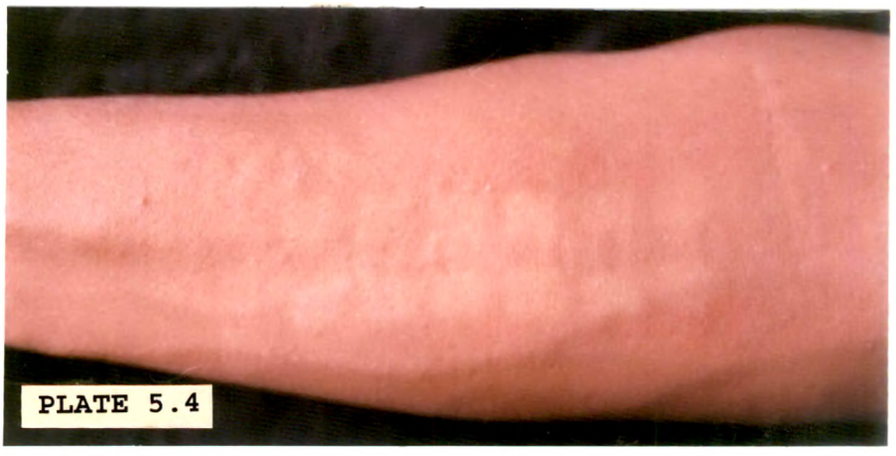


PLATE 5.4

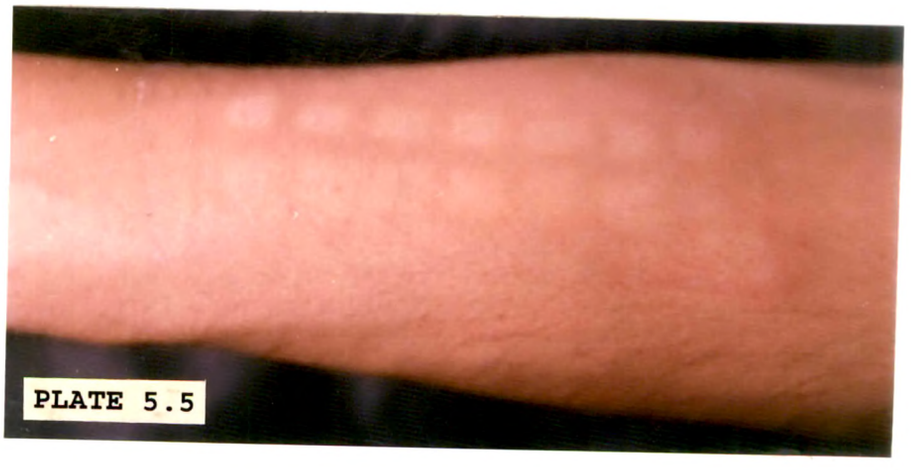


PLATE 5.5

response for a minute before allocating scores. The response was quantified on a 0-4 scale as follows(4) :

0 - normal skin
1 - slight pallor of indistinct outline
2 - more intense pallor with at least two corners of the application square outlined
3 - even pallor with a clear outline of the application area
4 - very intense pallor

The blanching responses of volunteers 1 and 3 to the formulations of set III and IV are shown in Plates 5.4 and 5.5 respectively.

The flowsheet for the sequential procedure for the skin blanching assay is shown in Fig.5.1.

For the purpose of this study, the formulations were divided into 4 sets (Table 5.1).

5.3 DATA ANALYSIS

For each set of experiments, the data was analysed on the following lines :

1. Comparison of the mean blanching scores of different volunteers for the formulations in the set to find out inter-volunteer variations. This was done using ANOVA (5a). If the calculated F value exceeded the table F value (5b), multiple comparisons were done using the Tukey's Multiple Range test (5c) to pin-point the volunteers differing significantly with respect to the blanching response.
2. Comparison of mean blanching scores for different formulations of a set on right and left hand to determine inter-hand variations. This was done using the student's t-test (5d).

3. Comparison of mean blanching scores of 3 observers for each observation in order to find out inter-observer variation. This was done using ANOVA.

4. Comparison of mean blanching scores for different formulations to rank the formulations in order of decreasing potency. If the calculated F value by ANOVA exceeded the table F value, multiple comparisons were done using the Dunnet's test (5e).

The significance levels for all the above comparisons were selected depending on the nature of comparison and are shown in their respective graphs.

5. For each formulation the data is presented in terms of the percentage of total possible score which was calculated using the following method(3) :

The maximum score per site	=	4
The number of independent observers	=	3
The number of sites per preparation per arm	=	1
The number of volunteers	=	8
Total possible score (TPS)	=	4x3x1x8

$$\text{Percent total possible score (\%TPS)} = \frac{\text{Actual score}}{\text{TPS}} \times 100$$

5.4 RESULTS AND DISCUSSIONS

Among the 42 volunteers who were screened for their blanching potential, only 8 volunteers gave an average blanching response. No marked variation in the blanching response was observed between the sexes. Volunteers below the age of 25 years failed to elicit a blanching response; however, not all volunteers above 25 years of age gave the response. Good results were not obtained when unoccluded study was performed and hence the idea was dropped.

The results obtained by applying the formulations of Set I on the forearms of 8 volunteers are shown in Figs. 5.3-5.7 and in Table 5.5. When the blanching scores between the volunteers were compared using ANOVA, the calculated F values were above the table F value at $P<0.05$, indicating that the volunteers differed significantly in their blanching potential. On further scrutiny with the Tukey's Multiple Range test, it was seen that the volunteer No.6 differed significantly in blanching potential as compared to volunteer No.2 and No.7 while volunteer No.3 differed significantly as compared to volunteer No.2. This must be due to the fact that people respond differently to compounds producing vasoconstriction.

When comparisons were made between the blanching scores for the right hand and those for the left hand (Fig.5.4) of 8 volunteers by the student's-t test, the calculated t value exceeded the table-t value only at $P<0.4$ which means that 40

TABLE 5.5

% TPS FOR THE SKIN BLANCHING ASSAY CONDUCTED ON FORMULATIONS OF SET I.

Formulation	% TPS
KTG	71.35
TPM	67.19
T_1	69.79
T_3	67.19
T_7	80.73
T_8	66.14
T_9	71.88
T_{10}	64.58
T_{11}	66.67
T_{17}	69.79
T_{21}	73.43
HPMC K4M gel base	5.20

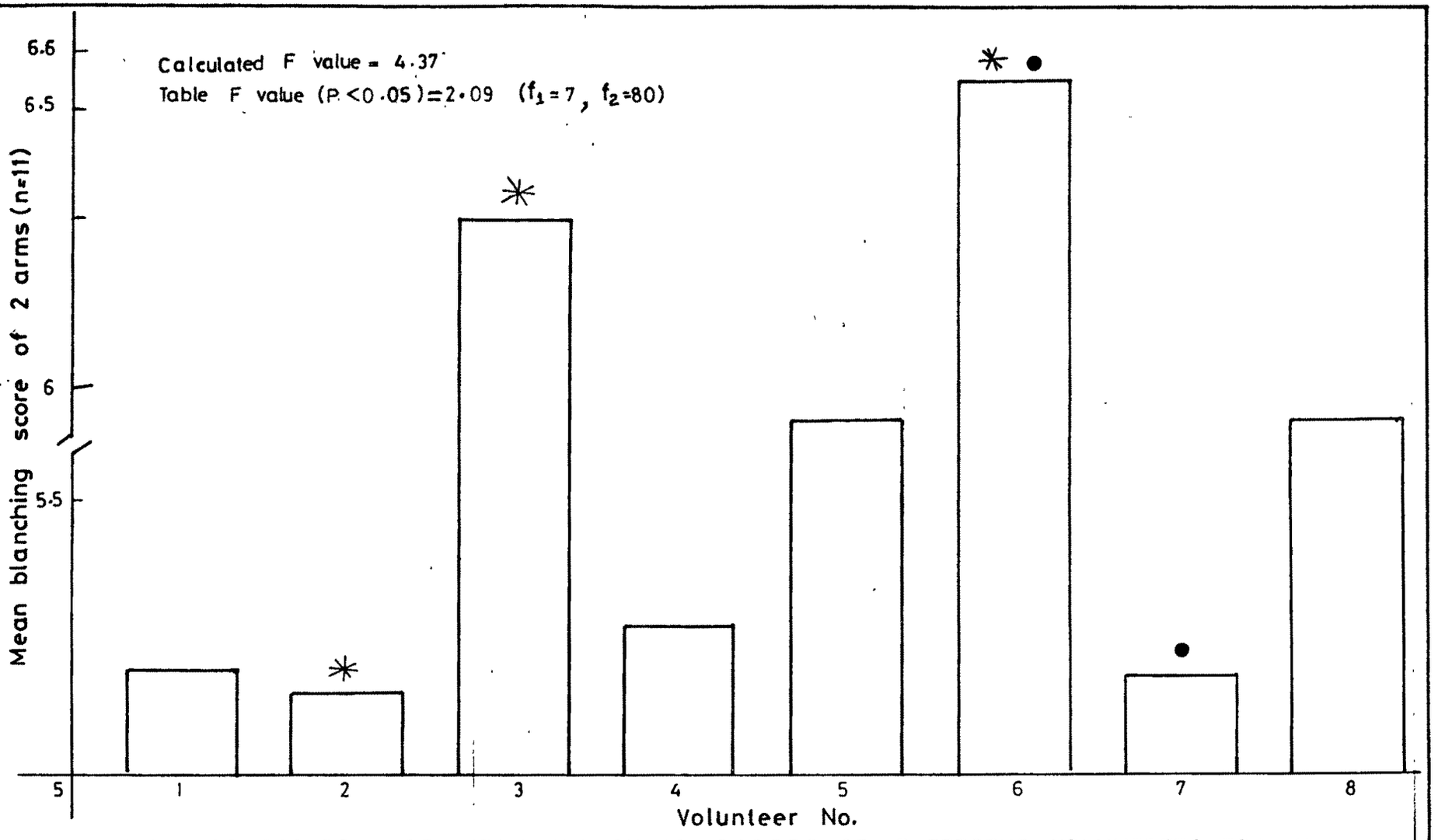


FIG. 5.3 : COMPARISON OF MEAN BLANCHING SCORES OF DIFFERENT VOLUNTEERS FOR 11 FORMULATIONS -SET I.

*, ● Volunteers differing significantly as per Tukey's Multiple Range Test

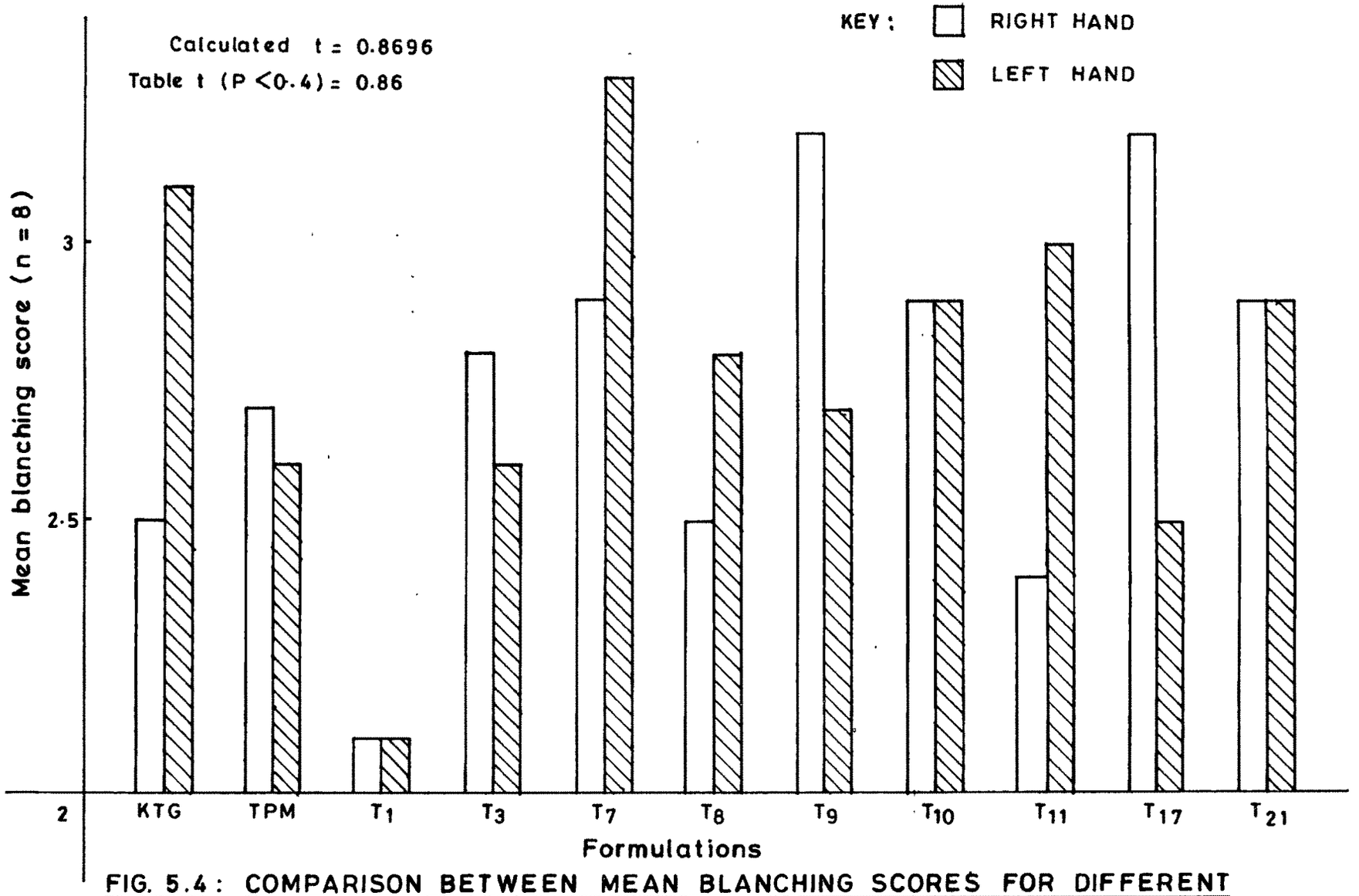


FIG. 5.4 : COMPARISON BETWEEN MEAN BLANCHING SCORES FOR DIFFERENT FORMULATIONS ON RIGHT HAND AND LEFT HAND - SET I.

out of 100 times the blanching scores on both arms differ i.e. the difference is not significant or the arm bias does not exist.

Since 3 observers were used for the study, it was very important to find out as to how they differ amongst themselves with respect to grading the blanching response. As seen from Fig.5.5, the calculated F value, for the blanching response of 8 volunteers for each formulation, is less than the table F value at $P<0.05$ indicating that no significant differences exist in the scores given by different observers for any of the formulations tested.

When the blanching scores for 8 volunteers for the 11 different formulations of Set I were compared using ANOVA (Fig. 5.6), the calculated F value was lower than the Table F value at $P<0.05$ indicating that the formulations compared, did not differ significantly from each other with respect to their skin blanching efficacy.

When the differences in the % TPS of the formulations of Set I were compared (Fig.5.7), except for formulations T_7, T_9 and T_{21}, all other formulations elicited blanching responses lower than those elicited by plain drug gel (KTG) with the order being $T_9 < T_{21} < T_7$ indicating that among the formulations tried, T_7 was the most potent as far as eliciting a blanching response was concerned.

From the blanching assay conducted on formulations of Set-I, it was obvious that among the liposomal batches

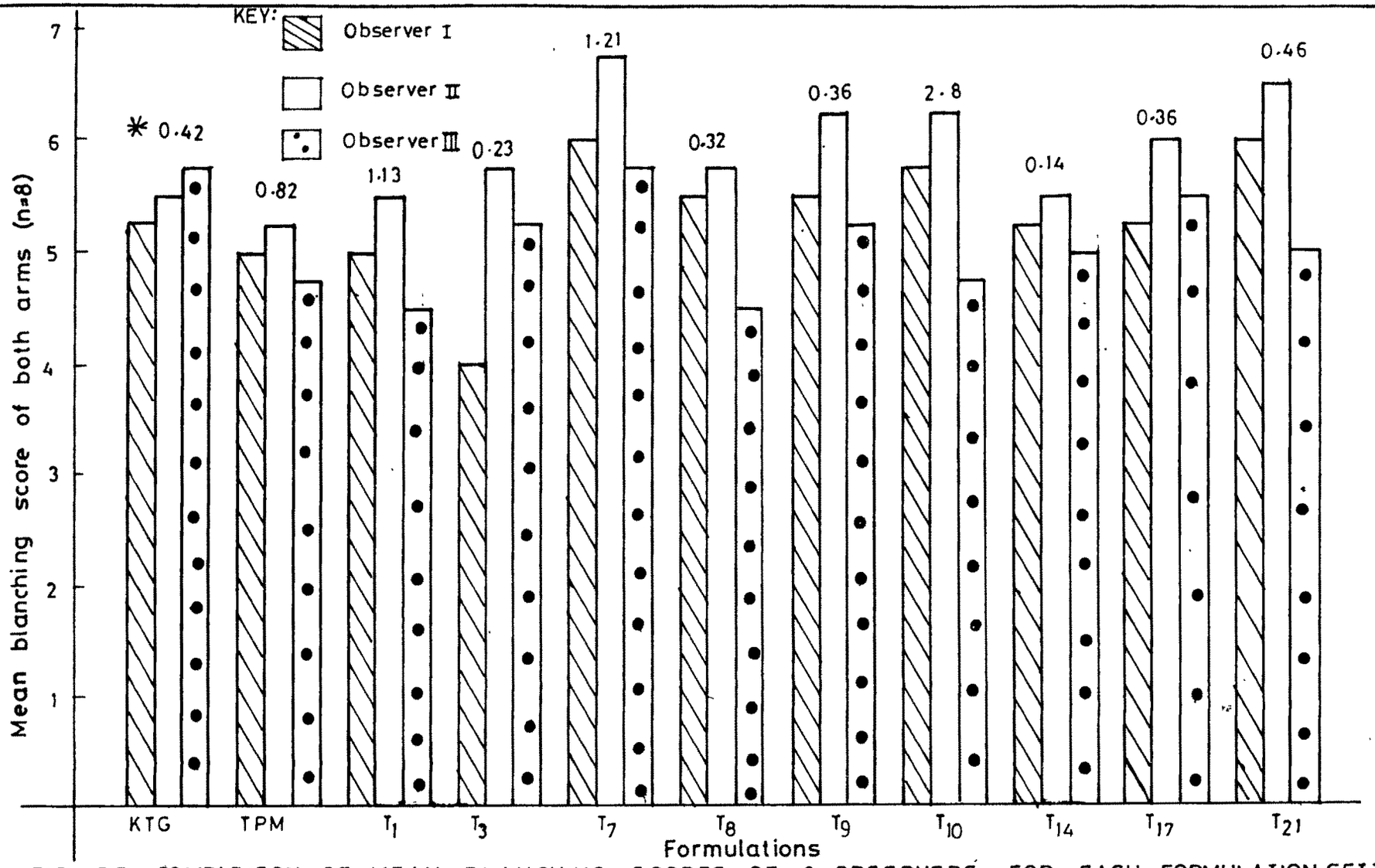


FIG. 5.5: COMPARISON OF MEAN BLANCHING SCORES OF 3 OBSERVERS FOR EACH FORMULATION-SET I

* Calculated F value for the formulation
(Table F value for $f_1=2$ and $f_2=21$ is 3.49 at $P<0.05$)

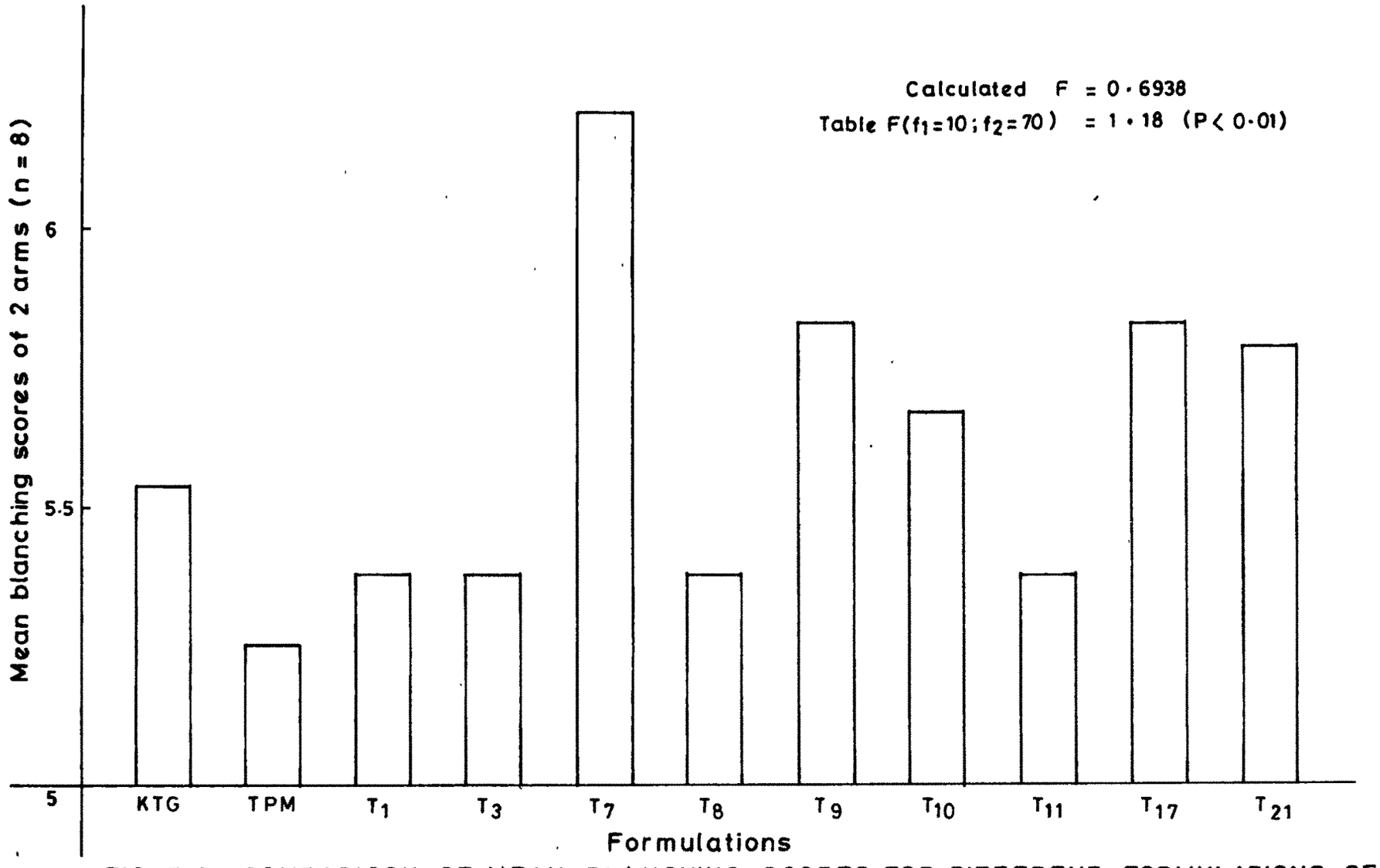


FIG. 5.6 : COMPARISON OF MEAN BLANCHING SCORES FOR DIFFERENT FORMULATIONS-SET I.

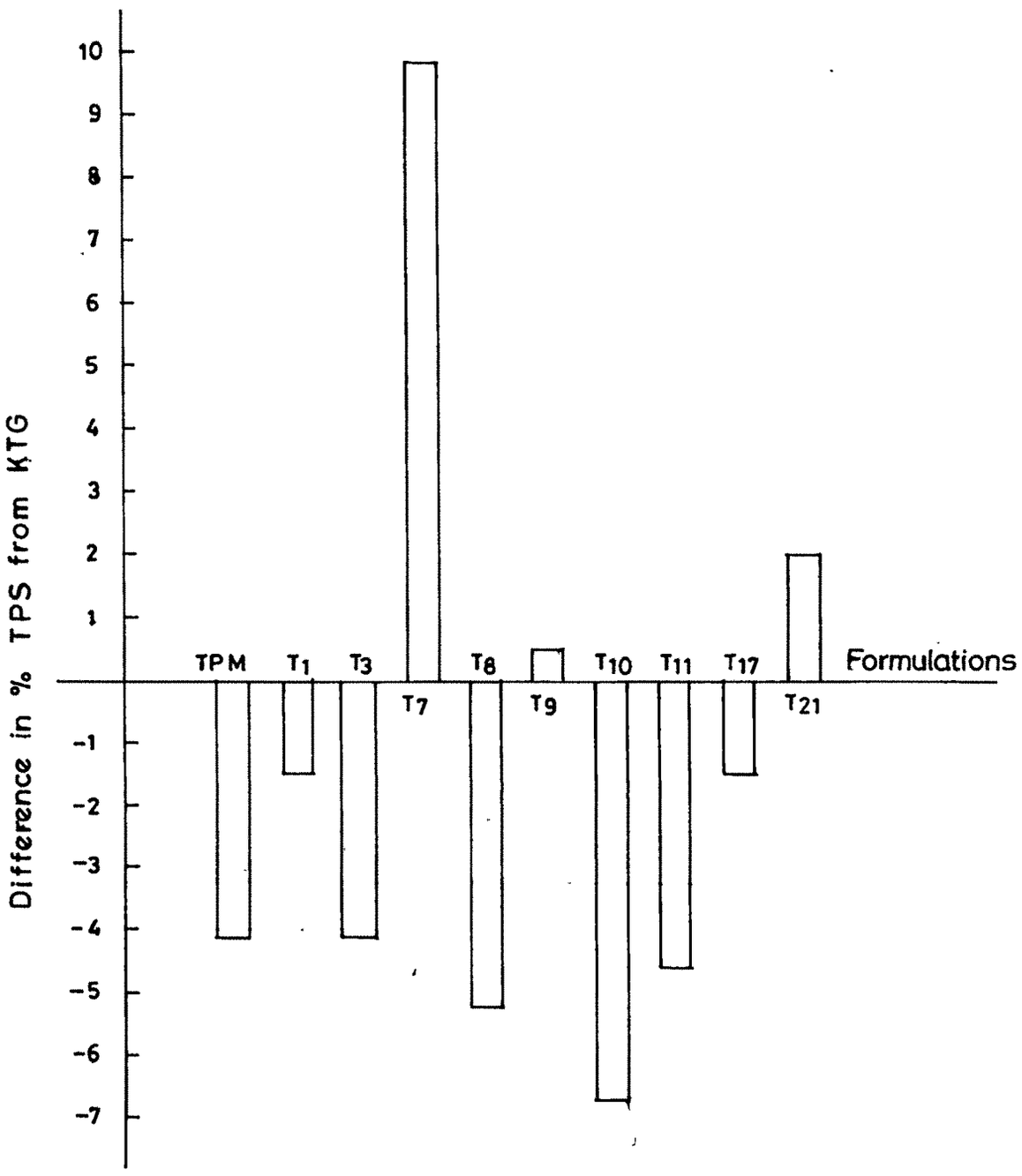


FIG. 5·7 : DIFFERENCE IN % TPS OF PHYSICAL MIXTURE GEL AND LIPOSOMAL GELS FROM % TPS OF KTG – SET I.

compared, the batch TRMA7 was found to be capable of eliciting the maximum blanching response and hence the liposomes of this batch were incorporated into different gel bases (Set II) to find out the effect of gel base on the blanching potential of liposomal TRMA. The results of Set II are shown in Figs. 5.8-5.12 and Table 5.6. As in case of Set I, in Set II also, the volunteers differed among themselves with respect to the intensity of eliciting the blanching response. Tukey's multiple Range test showed that the differences are significant between volunteers 4 and 8 and 3 and 8. However, when the blanching scores of right hand and left hand were compared (Fig.5.9) using the student's t-test, no significant difference was detected even at $P<0.2$.The observers too did not differ significantly in their blanching scores which is evident from the fact that the calculated F value for each formulation is less than the table F value at $P<0.05$. When the formulations of Set II were compared amongst themselves (Fig.5.11), the calculated F value exceeded the table F value at $P<0.01$ indicating a very significant difference in the formulations with respect to their abilities to elicit a blanching response. When the data was further scrutinized using the Dunnet's test, KLG, ELG and CLG were found to differ significantly from CTC (conventional cream) (Fig.5.11). Although the blanching responses produced by KTG, ETG, CTG and PLG exceed those of CTC (Fig.5.12) the differences were not statistically significant. This shows that incorporation of TRMA into HPMC K4M, HPMC E4M and

TABLE 5.6

% TPS FOR THE SKIN BLANCHING ASSAY CONDUCTED ON FORMULATIONS OF SET II.

Formulation	% TPS
KTG	63.50
ETG	59.88
CTG	54.13
PTG	44.25
ATC	33.38
CTC	47.88
KLG	84.88
ELG	85.38
CLG	72.88
PLG	63.00
HPMC K4M gel base	4.82
HPMC E4M gel base	5.01
Carbopol gel base	4.21
PVA gel base	6.34
Aqueous Cream base	2.32
CMC base	2.10

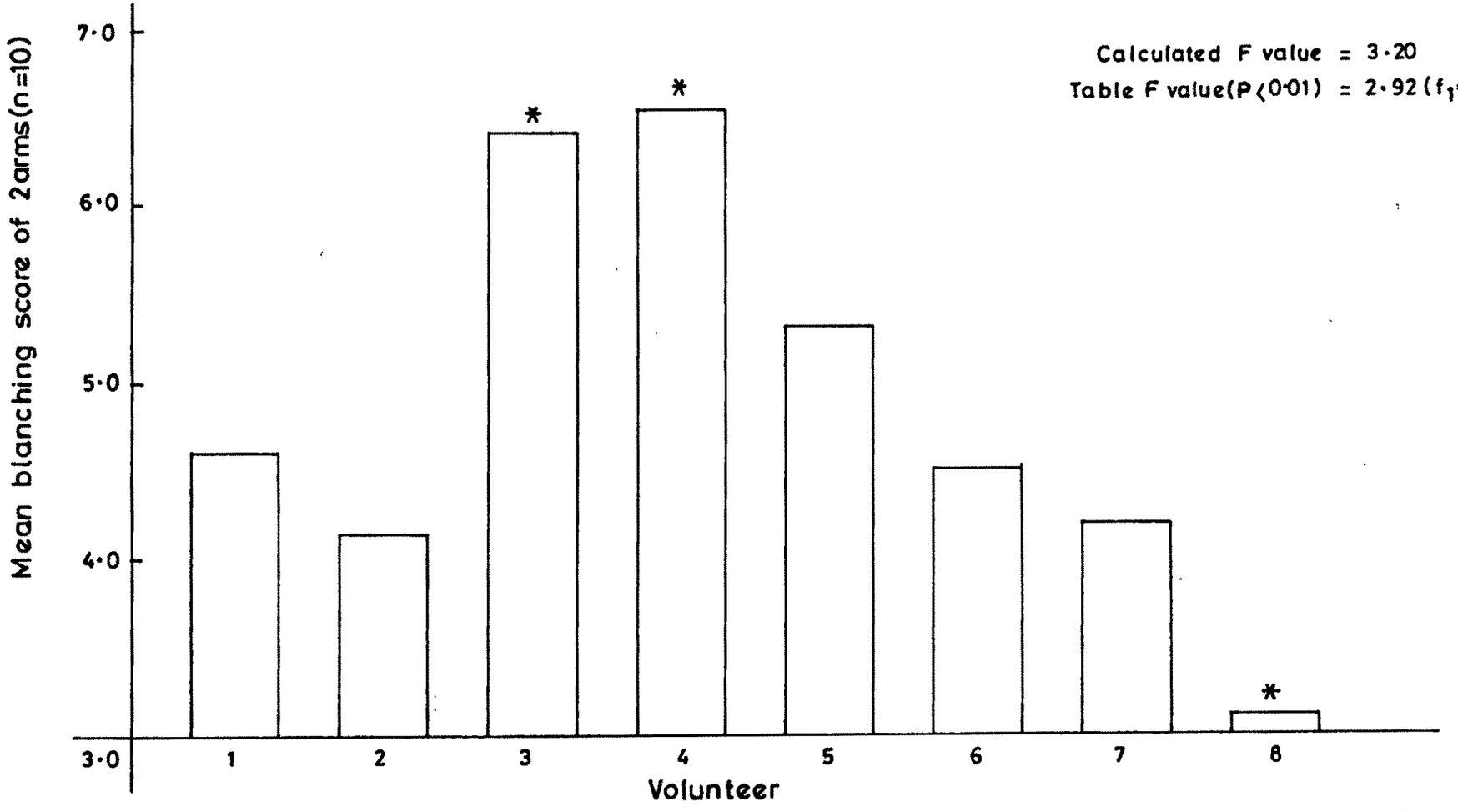


FIG. 5·8: COMPARISON OF MEAN BLANCHING SCORES OF DIFFERENT VOLUNTEERS FOR FORMULATIONS - SET II.

* Significantly differing volunteers as per Tukey's Multiple Range Test.

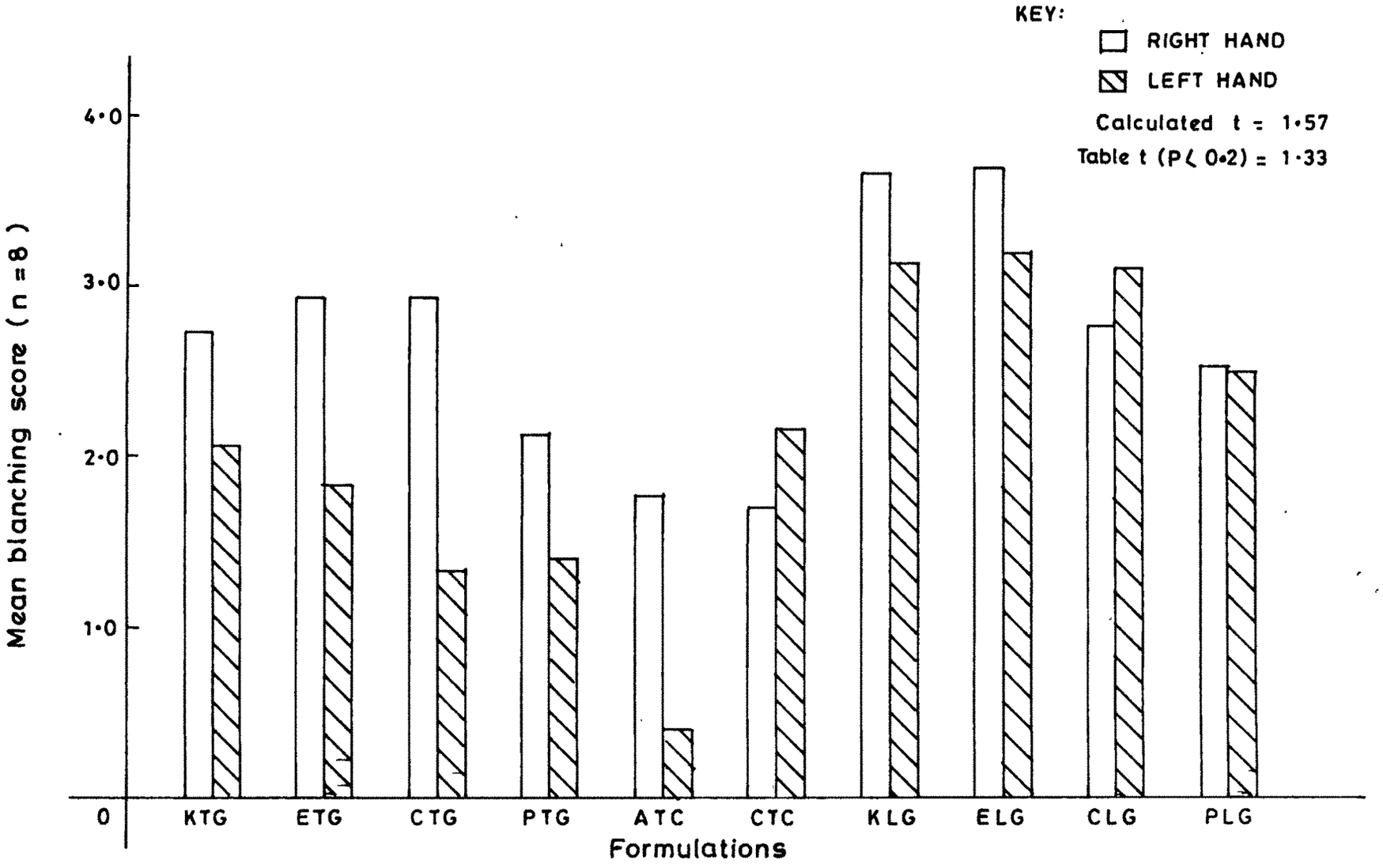


FIG. 5·9 : COMPARISON BETWEEN MEAN BLANCHING SCORES FOR DIFFERENT FORMULATIONS ON RIGHT HAND AND LEFT HAND - SET II.

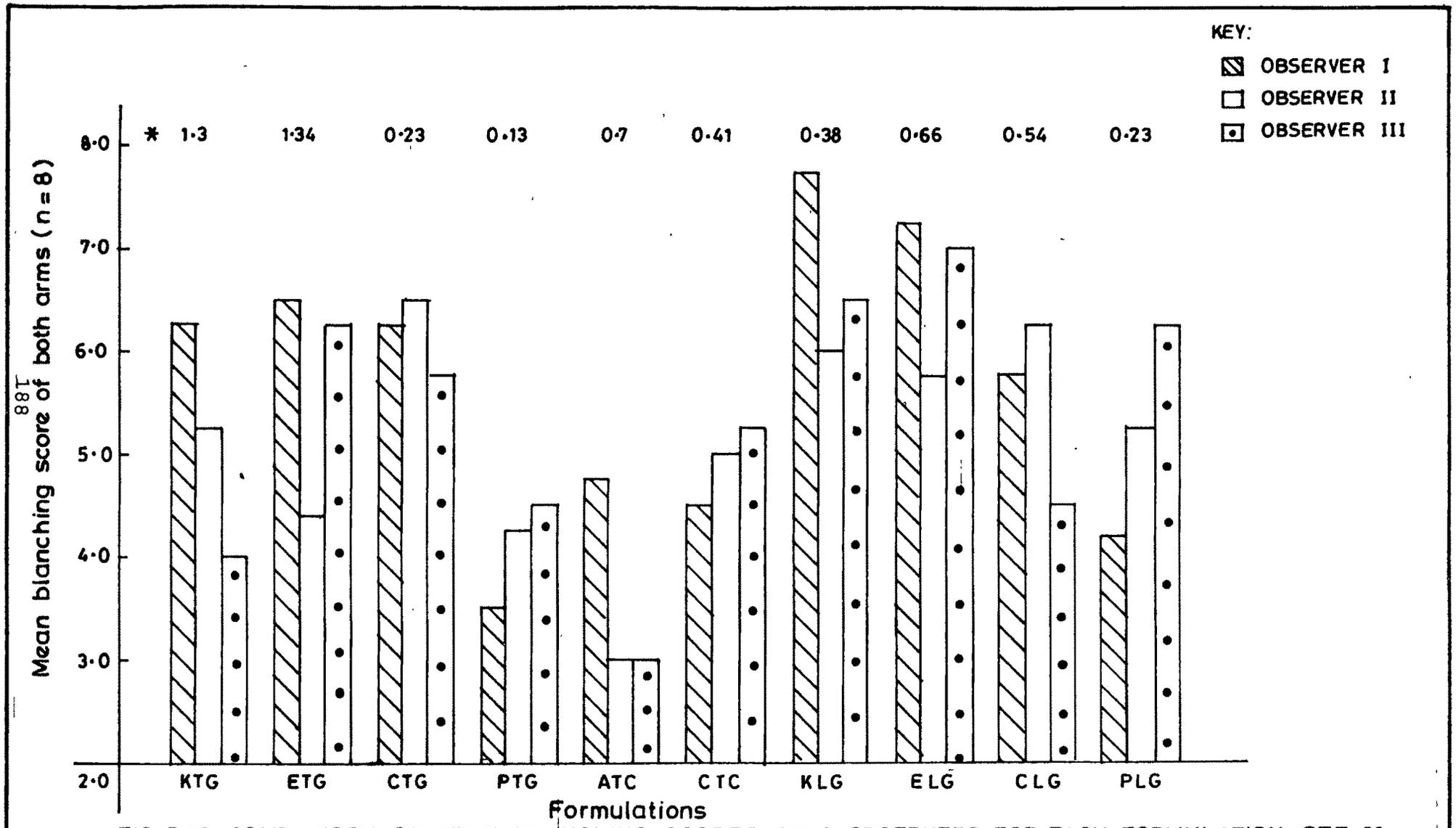


FIG. 5·10 : COMPARISON OF MEAN BLANCHING SCORES OF 3 OBSERVERS FOR EACH FORMULATION - SET II.

* Calculated F value for the formulation
(Table F value for $f_1=2$; $f_2=21$ is 3·49 at $P<0·05$)

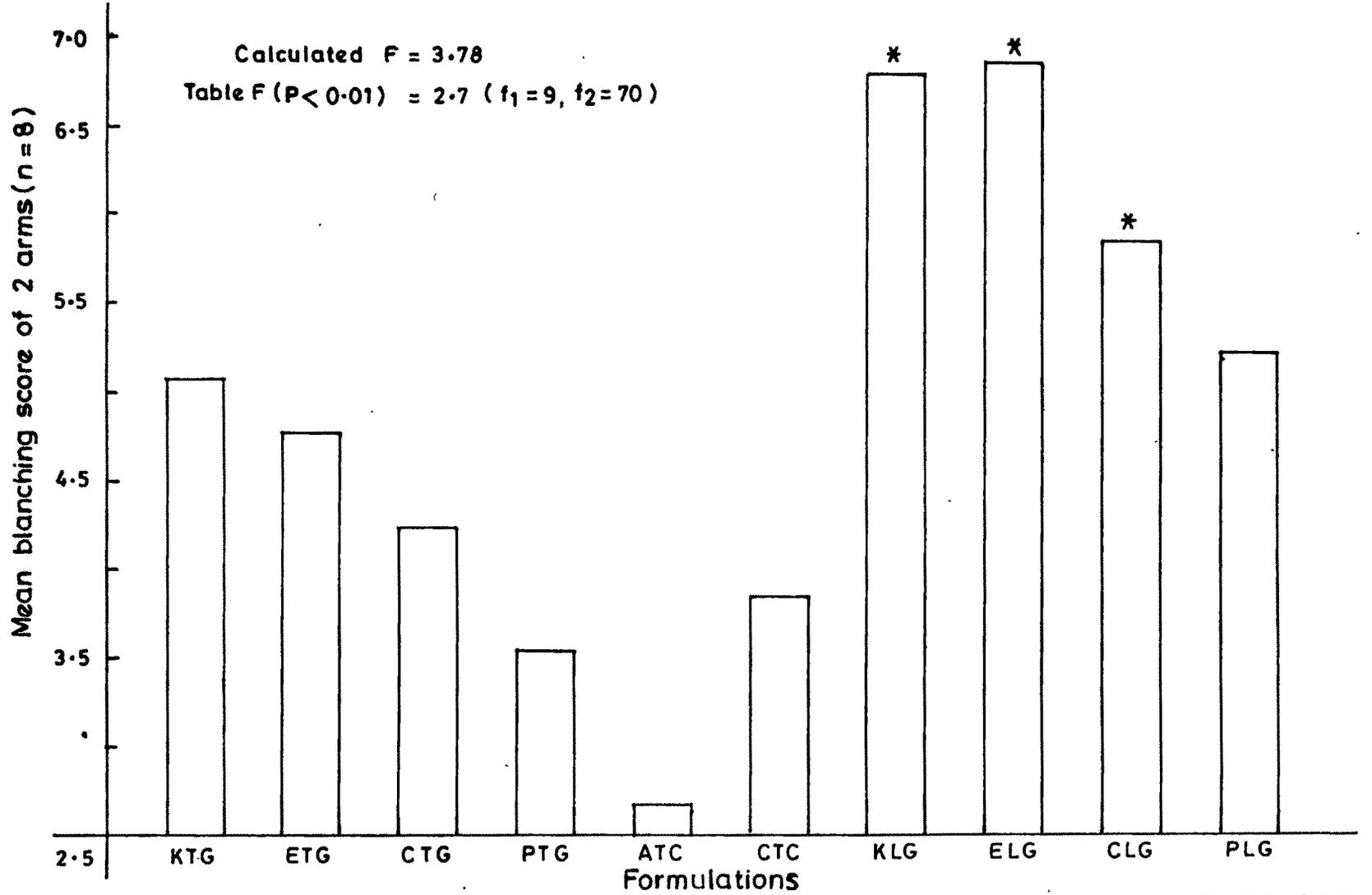


FIG. 5·11 : COMPARISON OF MEAN BLANCHING SCORES FOR DIFFERENT FORMULATIONS–SET·II.

* Formulations significantly different from CTC as per Dunnet's test

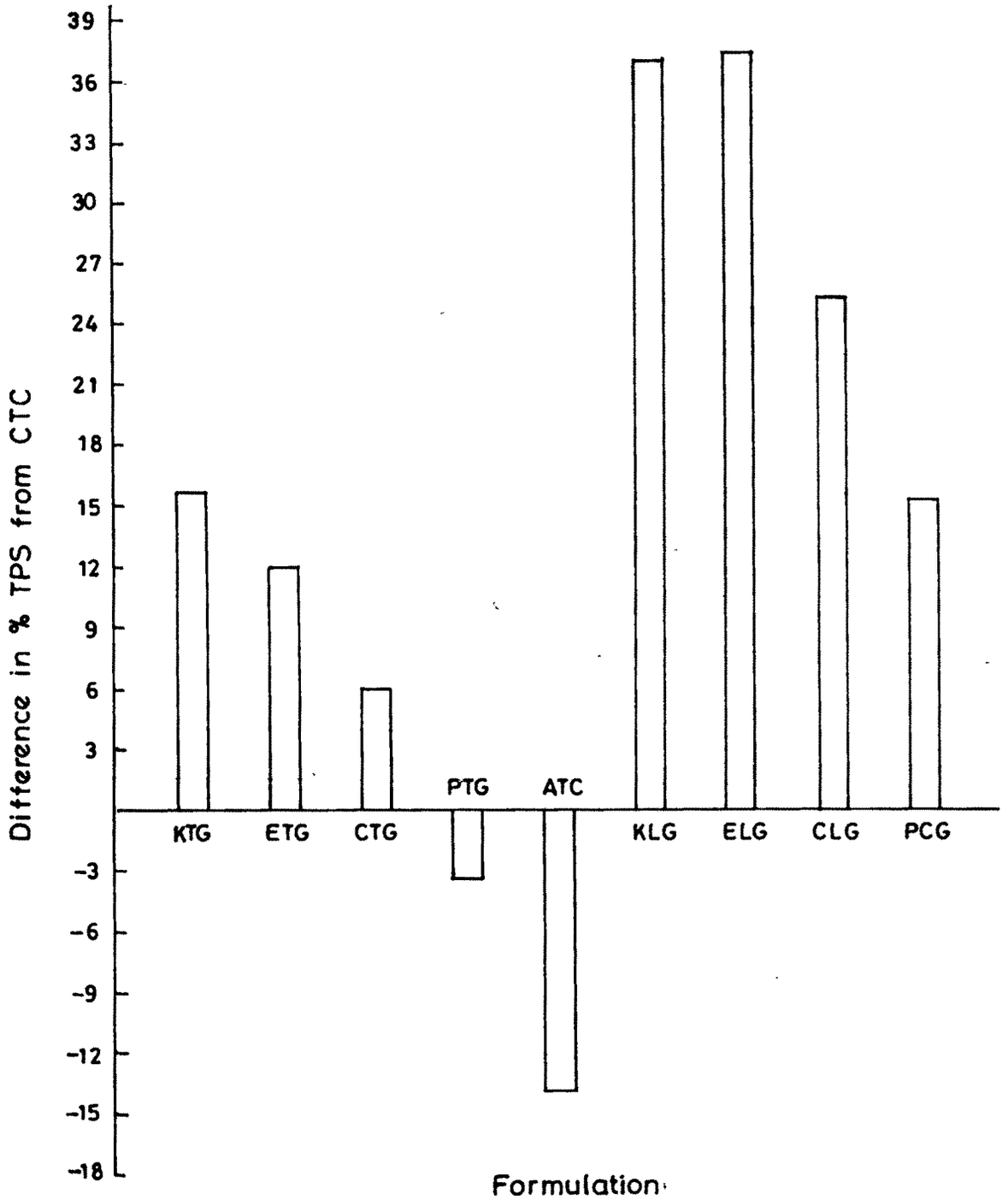


FIG. 5·12 : DIFFERENCE IN THE % TPS VALUES OF FORMULATIONS OF SET II FROM THAT OF CTC.

carbopol 941 gel bases may give efficacy slightly higher (not statistically significant) than that when it is incorporated in the conventional Cetomacrogol cream base. Besides, incorporation of liposomal TRMA in the above mentioned gel bases may improve the efficacy further. Although incorporation of liposomal TRMA into PVA gel base gives efficacy comparable to that of TRMA in Cetomacrogel cream base, plain TRMA in PVA gel base gives efficacy lower than that of CTC indicating that this base hampers the blanching response which may be due to hampering the passage of the drug from gel to the skin surface.

Results of the blanching assay performed using the formulations of Set III are shown in Table 5.7 and Figs. 5.13-5.17. For this set, the calculated F value is less than the table F value at $P<0.05$ (Fig.5.13) when the response given by different volunteers is compared indicating that the volunteers do not differ significantly with respect to blanching response for FLU gel/cream. The blanching scores, when compared for right and left arm, are not significantly different even at $P<0.4$ using the student's t-test (Fig.5.14). The 3 observers do not differ significantly in their assignment of blanching scores since the calculated F value is lower than the Table F value at $P<0.05$ (Fig.5.15). Comparing the different formulations of FLU for their ability to elicit a blanching response (Fig.5.16), the calculated F value exceeds the Table F value at $P<0.05$ but not at $P<0.01$ indicating that the differences are less significant. When

TABLE 5.7

% TPS FOR THE SKIN BLANCHING ASSAY CONDUCTED ON FORMULATIONS OF SET III AND SET IV.

Set	Formulation	% TPS
III	CFC	72.25
	KFG	88.02
	FPM	78.65
	F_1	91.15
	F_2	92.19
	F_3	80.21
	HPMC K4M gel base	1.00
	CMC base	0.50
IV	CCC	70.34
	KCG	81.25
	CPM	76.04
	C_1	81.77
	C_2	89.58
	C_3	88.01
	HPMC K4M gel base	1.00
	CMC base	0.50

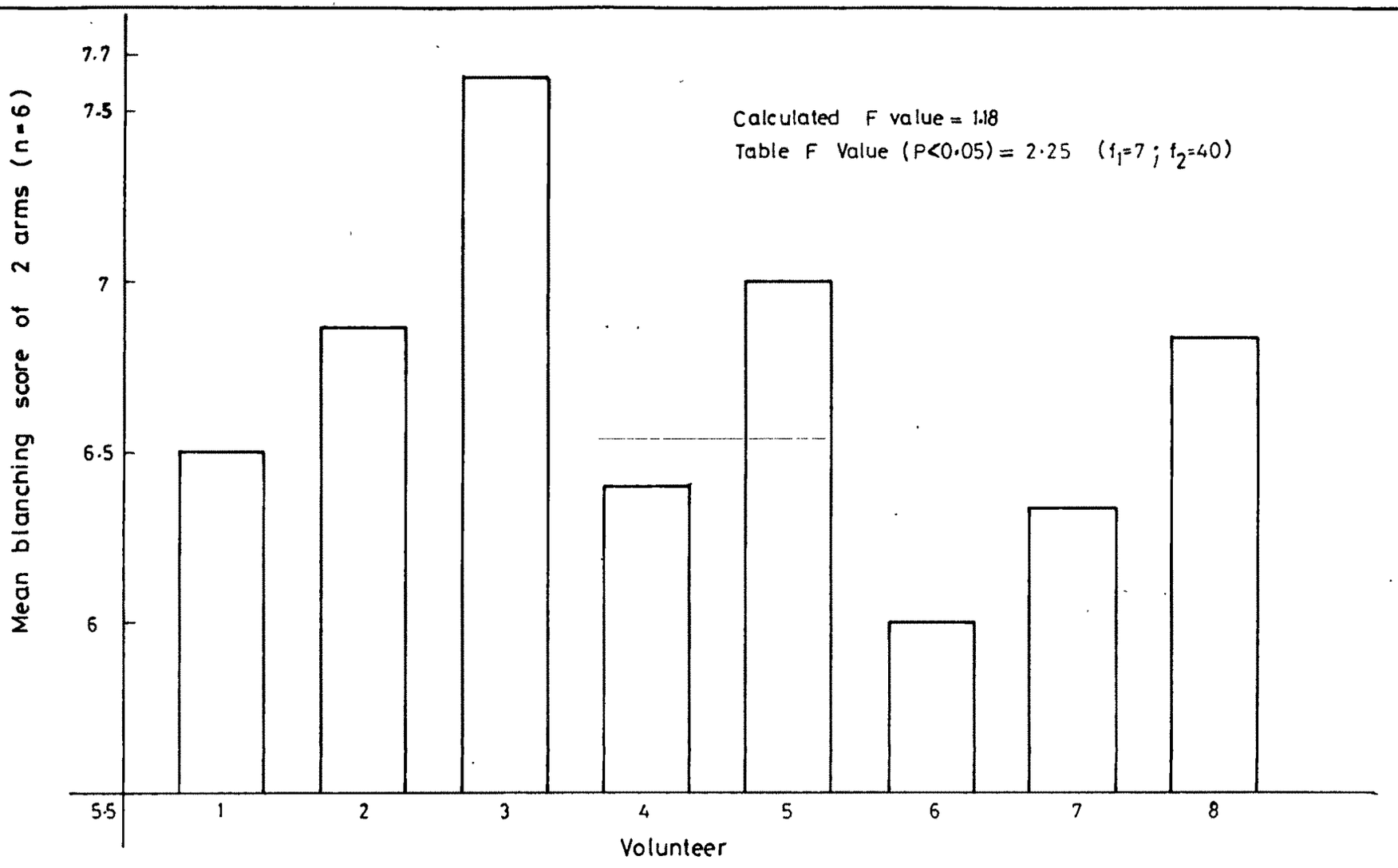


FIG. 5.13 : COMPARISON OF MEAN BLANCHING SCORES OF DIFFERENT VOLUNTEERS FOR FORMULATIONS OF FLU - SET III.

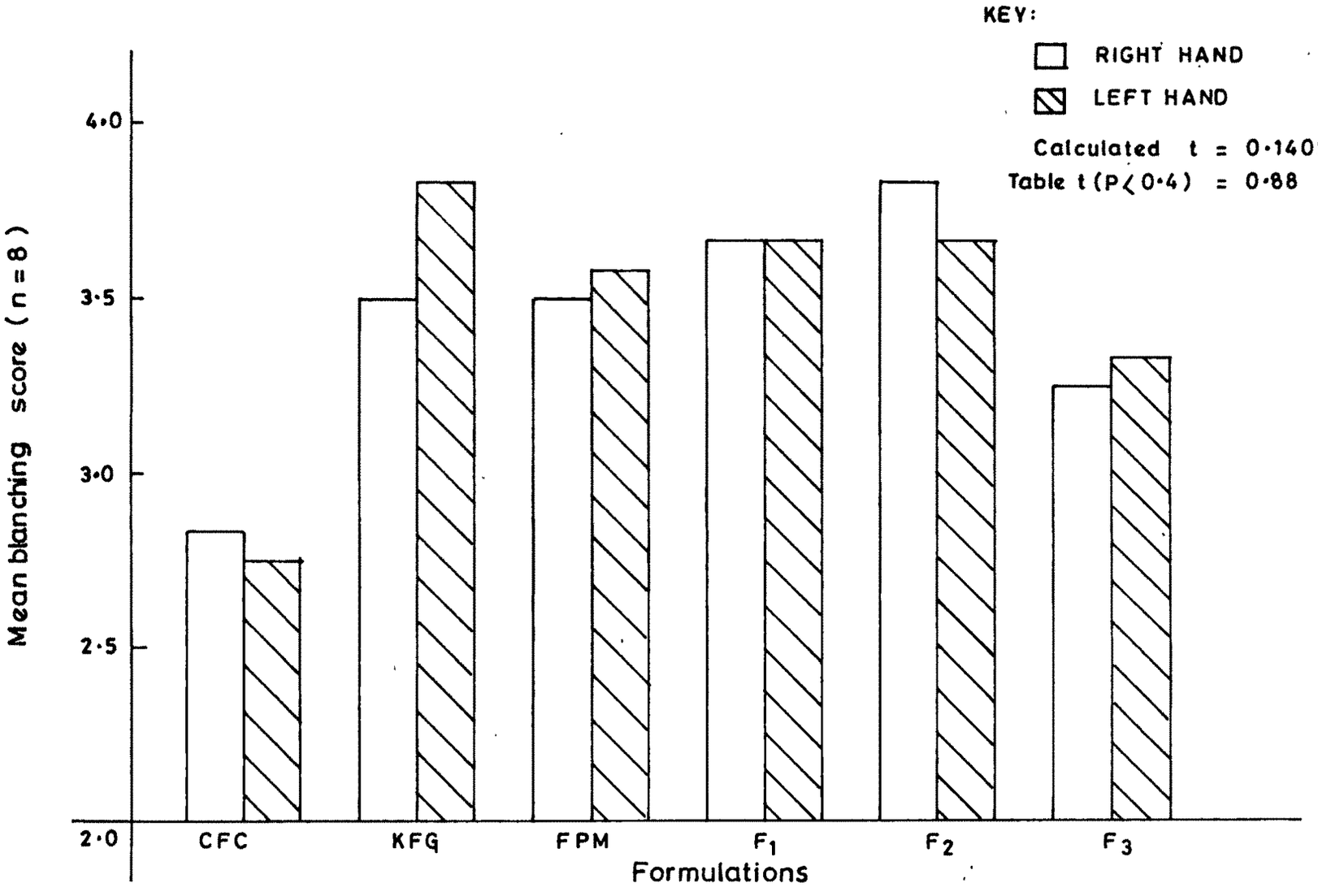


FIG. 5·14: COMPARISON BETWEEN MEAN BLANCHING SCORES FOR FLU FORMULATIONS ON RIGHT HAND AND LEFT HAND - SET III.

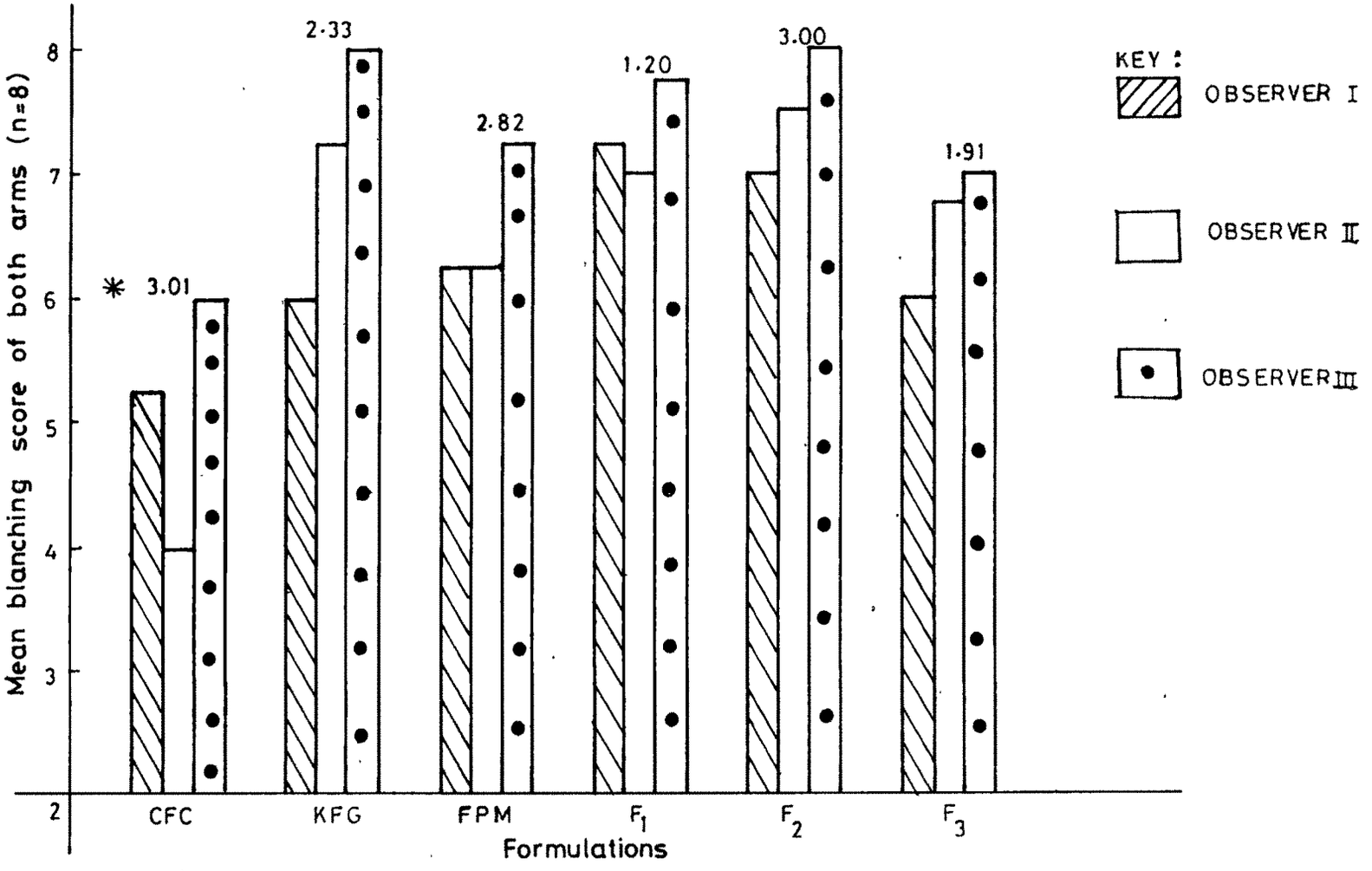
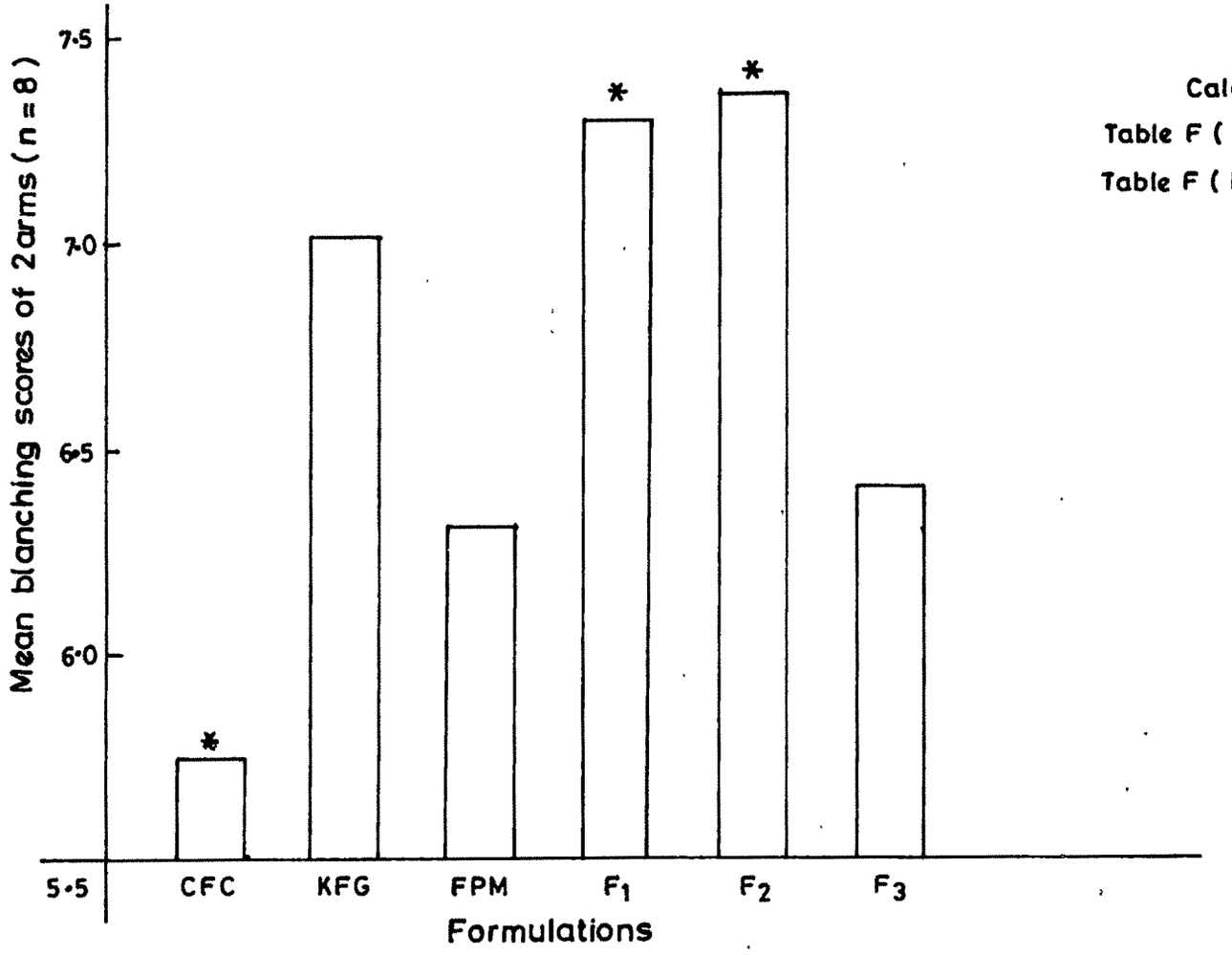


FIG. 5.15 : COMPARISON OF MEAN BLANCHING SCORES OF 3 OBSERVERS FOR FLU FORMULATIONS-SET III.

* Calculated F value for the formulation (Table F value for $f_1 = 2$ & $f_2 = 21$ is 3.49 at $P < 0.05$)



Calculated F = 3·14

Table F (P < 0.01) = 3·51 ($f_1 = 5$; $f_2 = 42$)

Table F (P < 0·05) = 2·45

FIG. 5·16: COMPARISON OF MEAN BLANCHING SCORES FOR FLU FORMULATIONS - SET III.

* Formulations significantly different as per Dunnet's test (P ≤ 0·05)

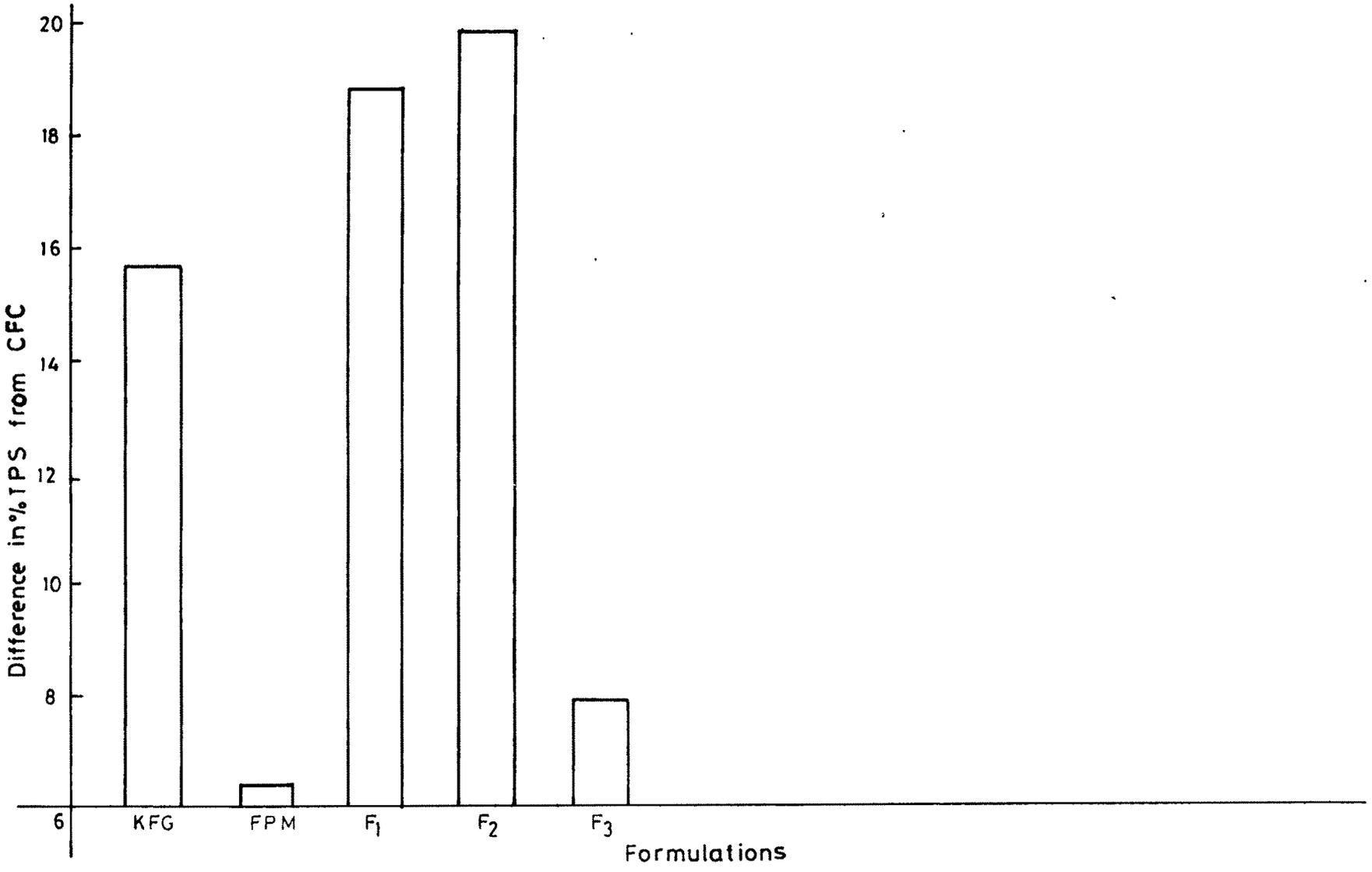


FIG. 5.17 : DIFFERENCE IN THE % TPS OF FLU GELS FROM THAT OF CFC – SET III.

the various formulations were compared with CFC (conventional formulation) using the Dunnet's test at $P<0.05$, F_2 and F_3 differed significantly. All the FLU formulations tested elicited a blanching response greater than that elicited by CFC (Fig.5.17) the order being $FPM<F_1<KFG<F_3<F_2$.

Results of the blanching assay performed using the formulations of Set IV are shown in Table 5.7 and Figs. 5.18-5.22. When the blanching response of different volunteers to formulations of this set was compared (Fig.5.18), the calculated F value was less than the Table F value at $P<0.05$ indicating that the volunteers do not differ significantly with respect to giving a blanching response for formulations of CLO tested. The fact that the calculated t-value exceeds the Table-t value only at $P<0.4$ (Fig.5.19) indicates that there is no significantly significant difference in the blanching responses obtained between the right and left arms. The 3 observers did not differ significantly in their assignment of blanching scores since the calculated F value is lower than the Table F value at $P<0.05$ (Fig.5.20). Although all the formulations tested elicited a blanching response greater than that elicited by CCC (conventional cream) (Fig.5.22), the difference was not statistically significant since the calculated F value did not exceed the Table F value at $P<0.05$ (Fig.5.21).

From the results of the skin blanching assay conducted on all the 4 sets, it is seen that differences are observed in between the volunteers for formulations of TRMA but not

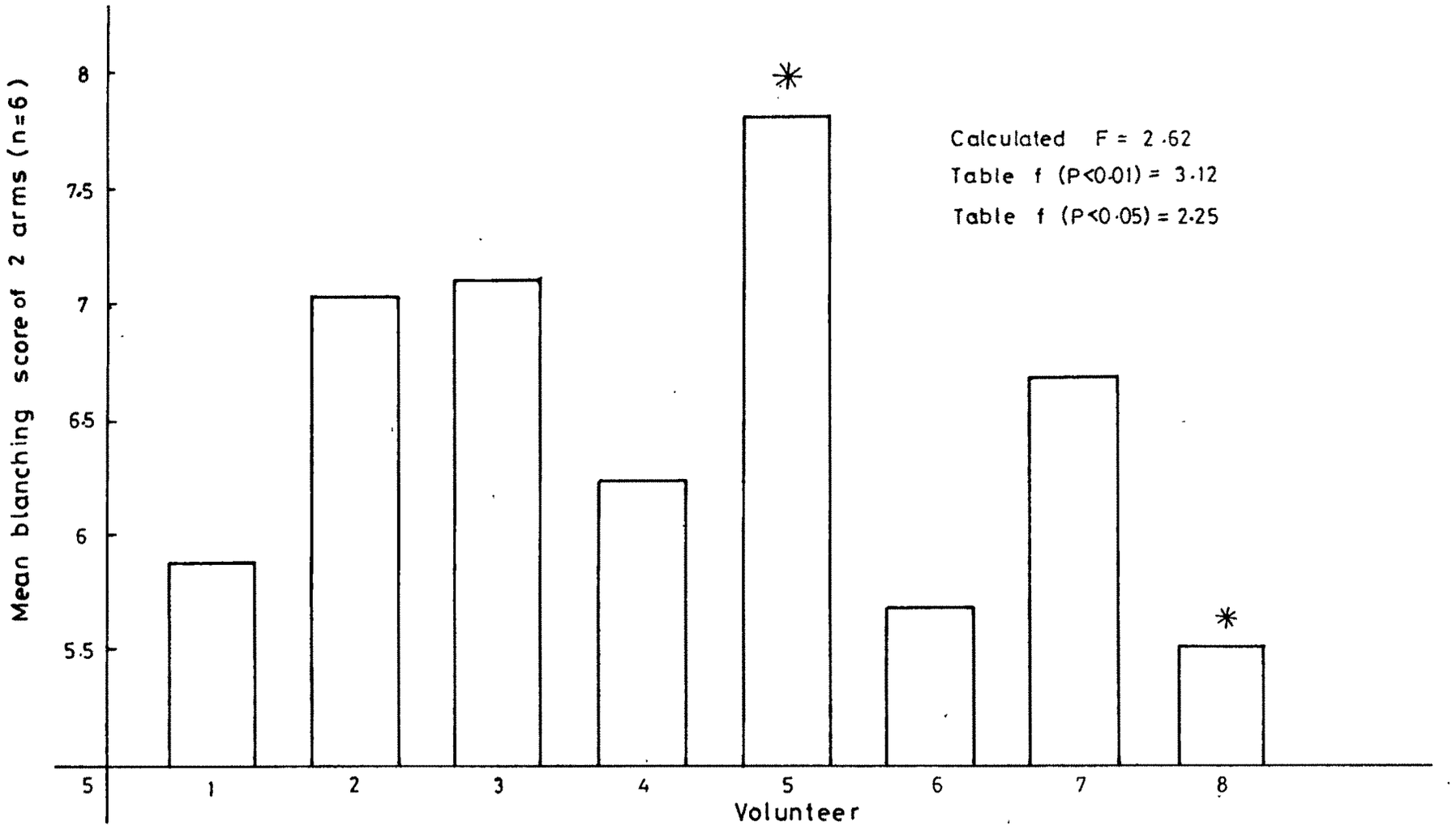


FIG. 5.18: COMPARISON OF MEAN BLANCHING SCORES OF DIFFERENT VOLUNTEERS FOR FORMULATIONS OF CLO - SET IV.

* Significantly different volunteers as per Tukey's multiple range test.

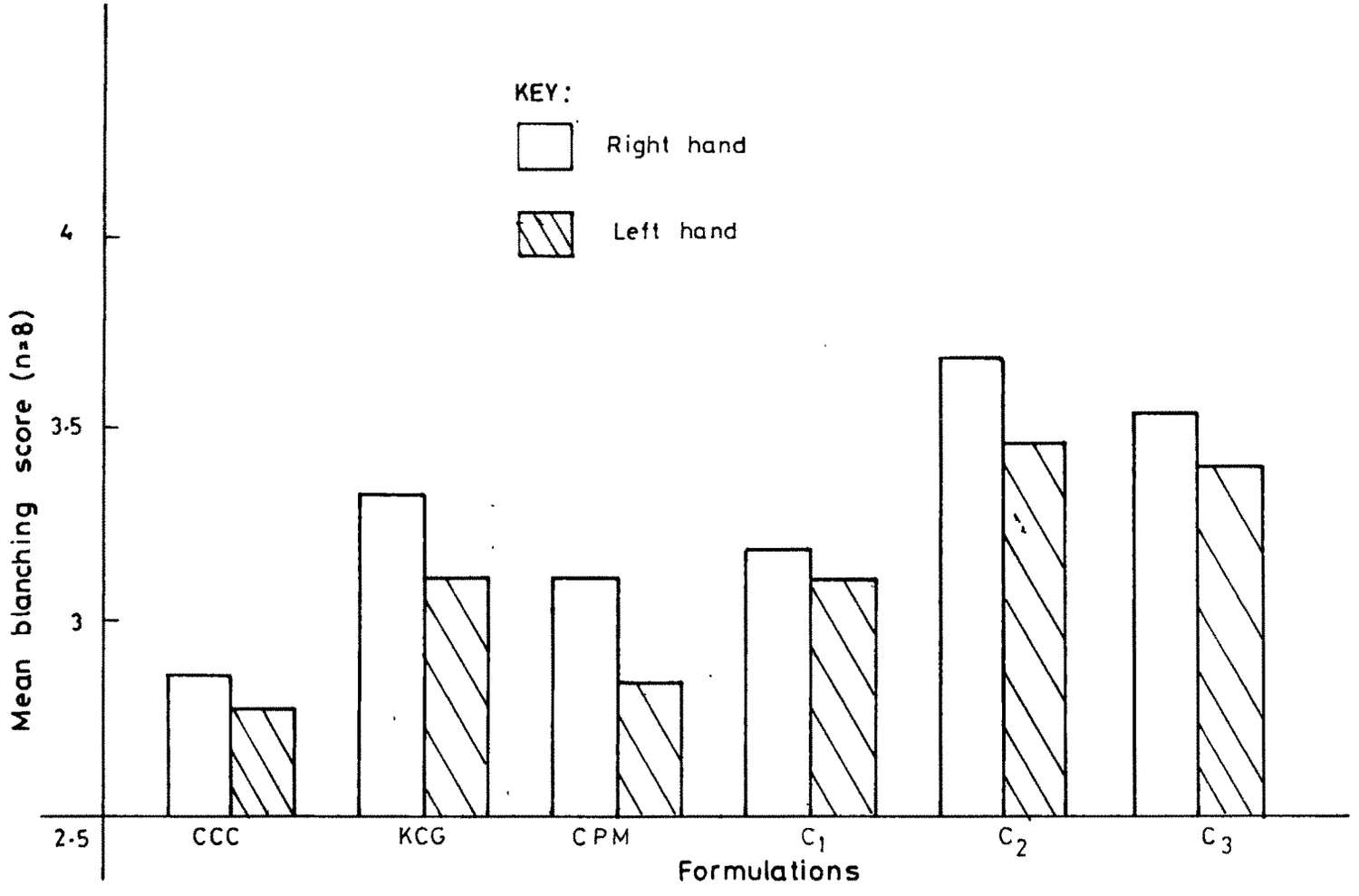


FIG. 5·19 : COMPARISON BETWEEN MEAN BLANCHING SCORES FOR CLO FORMULATIONS ON RIGHT HAND AND LEFT HAND - SET IV .

Calculated t = 0·989

Table t (P<0.4) = 0·88

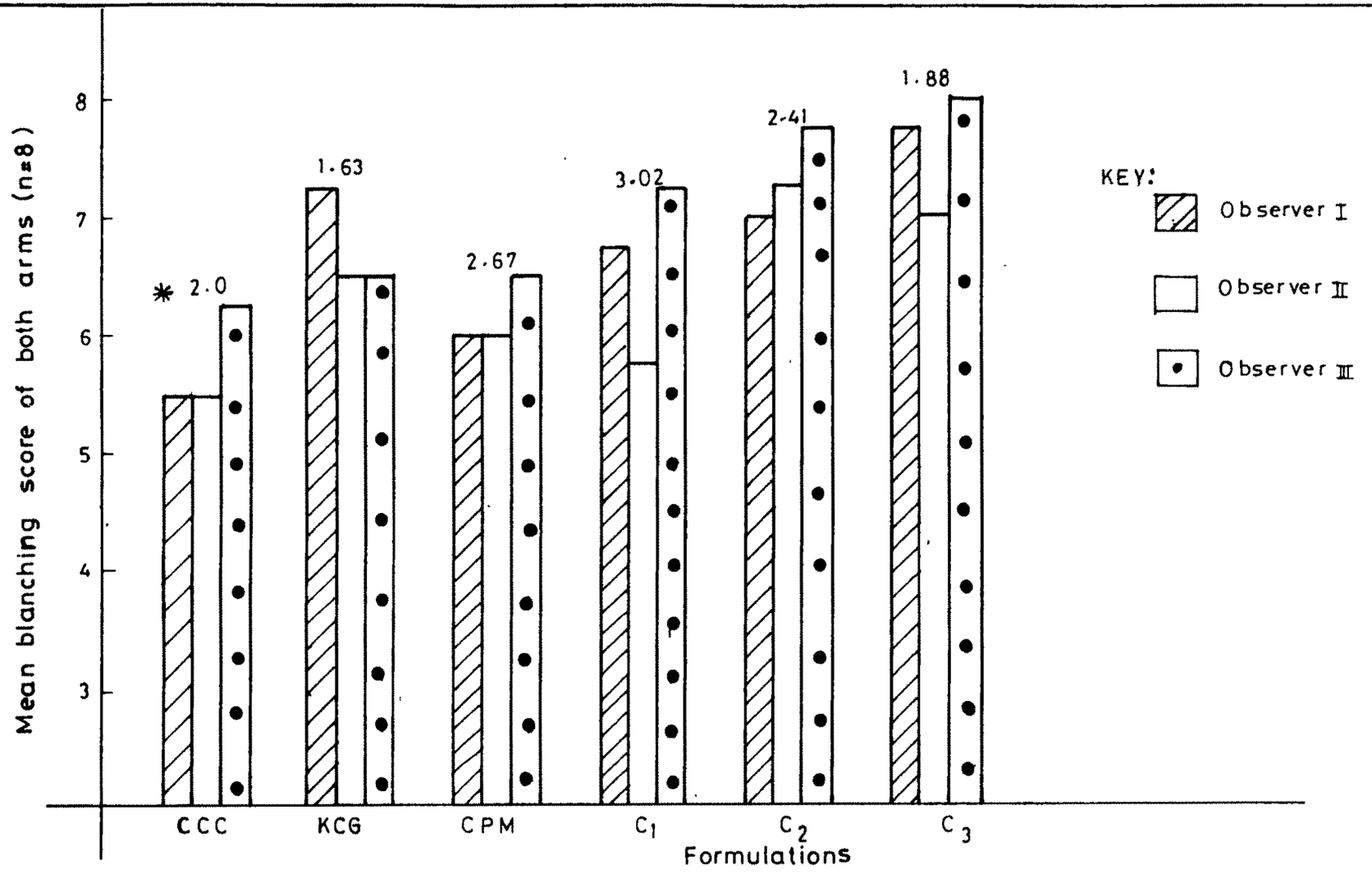


FIG. 5.20: COMPARISON OF MEAN BLANCHING SCORES OF 3 OBSERVERS FOR CLO FORMULATIONS-SET IV

* Calculated F value for the formulation
(Table F value for f_1 =2 & f_2=21 is 3.49 at $P<0.05$)

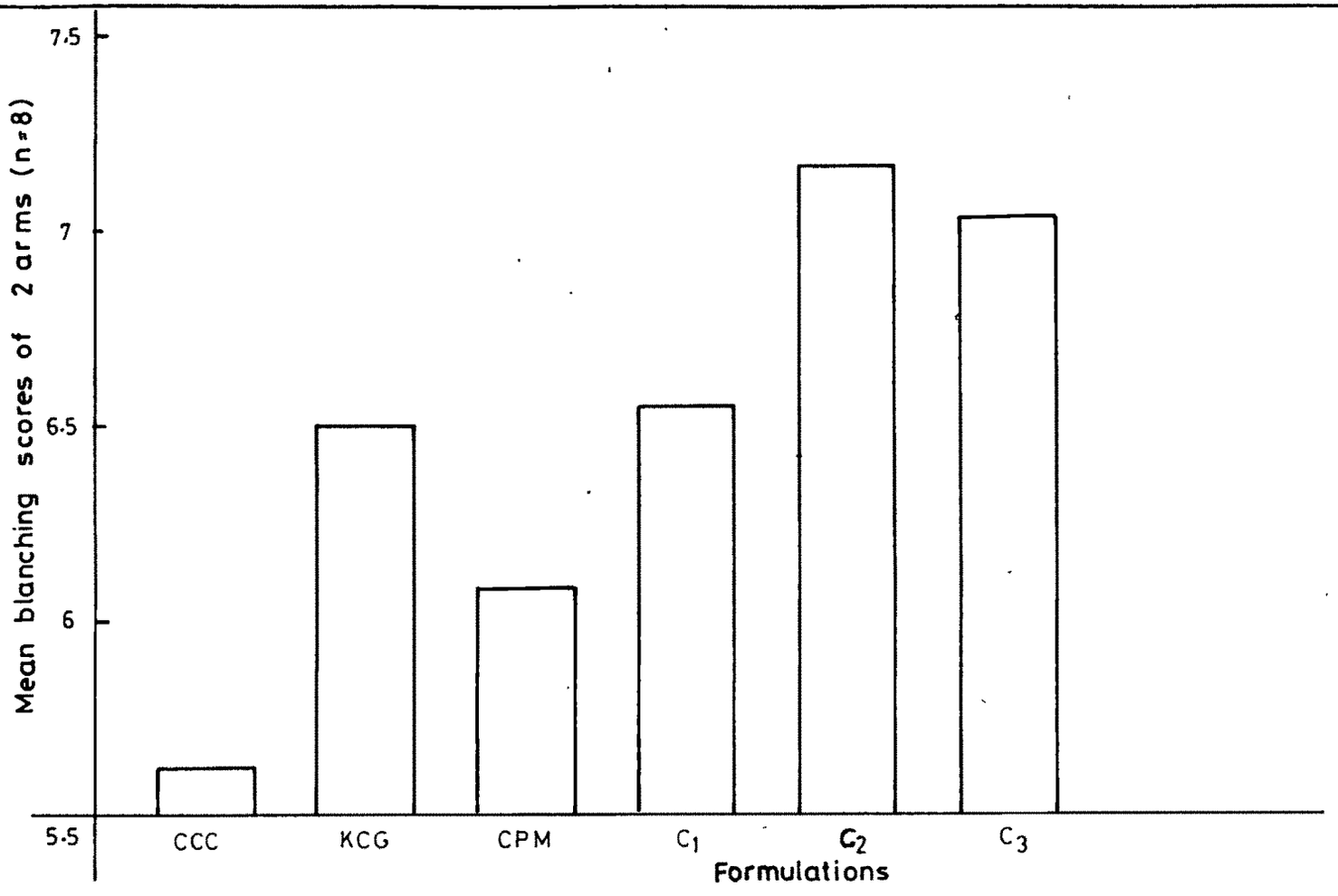


FIG. 5.21: COMPARISON OF MEAN BLANCHING SCORES FOR CLO FORMULATIONS - SET IV.

Calculated $F = 1.55$

Table F ($P<0.05$) $= 2.45$ ($f_1=5$, $f_2=42$)

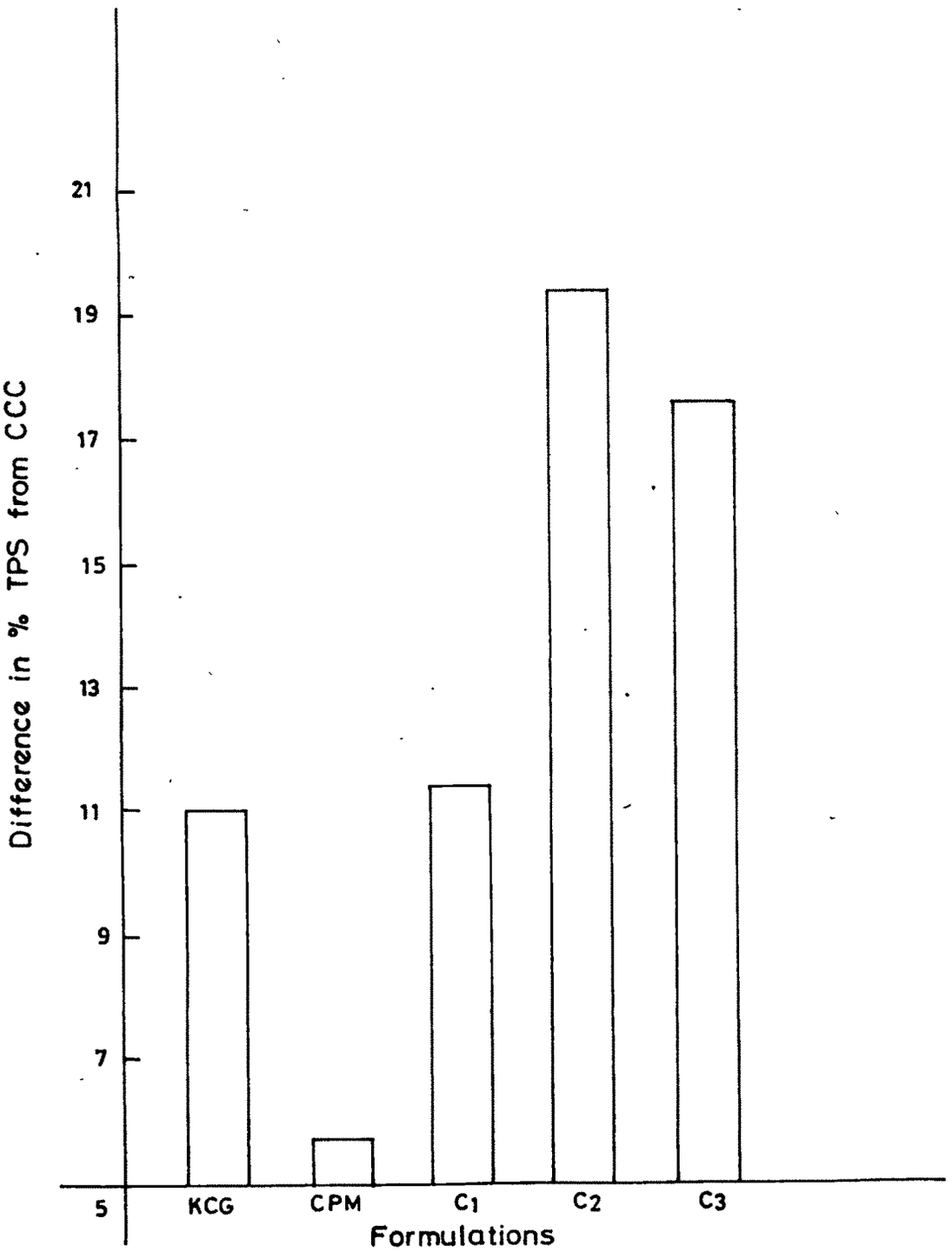


FIG. 5·22 : DIFFERENCE IN THE % TPS OF CLO GELS FROM THAT OF CCC - SET IV

for those of FLU and CLO. This must be because FLU and CLO are more potent than TRMA and hence the distinguishing ability of the volunteer is off-set by the high ability of the drug to induce blanching. No significant differences exist in the blanching scores of right and left arms for all the formulations tested during this study indicating that there is no arm bias for the blanching response. The observers did not differ significantly with respect to grading the blanching response indicating that the observer panel was unanimous in their grading. Liposomal formulations of TRMA did not differ significantly amongst themselves with respect to their ability to elicit the blanching response with T_7 giving the maximum response. Among the bases tried, liposomal TRMA in HPMC K4M gel base (KLG), gave the best blanching response as compared to TRMA in Cetomacrogol cream base. For liposomal FLU formulations, F_1, F_2 and F_3 elicited a significantly higher response as compared to that by FLU in Cetomacrogol cream base while for liposomal CLO formulations, C_2 elicited the highest response as compared to that by CLO in Cetomacrogol cream base.

Hence, formulations T_7, F_2 and C_2 were selected, as ideal, for conducting clinical trials.

5.5 REFERENCES

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