C H A P T E R - IV

PHARMACOLOGICAL STUDY FOR THE ANTIFERTILITY ACTION OF *CARICA PAPAYA* SEEDS

ABSTRACT

An attempt has been made to standardize the antifertility inducing fraction of *Carica papaya* seed extract and then the active principle, vis-a-vis biological activity against rats.

INTRODUCTION

Carica papaya Linn. (Papaya, Papaw tree) belongs to the family Caricaceae and is sub-herbaceous tree native of tropical Central America.[1]

It generally grows to a height of 10-25 ft. with a crown like large deeply lobed leaves giving a palm like appearance. Plant is normally dioecious but occasionally monoecious; hermaphrodite flowers have also been recorded.[2] The fruits are borne near the top of trunk, closely packed at the bases of the leaves.

Presently tree is grown in all tropical and subtropical regions of India; and its parts like leaves, fruits and seeds are utilized for medicinal purpose.[3]

The leaves have anthelmintic and febrifuge properties. They are used in beri-beri.[4]

The green fruit is laxative, diuretic, while its juice acts as an emmenagogue and in large dose as ecbolic.[3] The latex of green fruit has been reported to posses oxytocic activity of high order. Pulp extract of petroleum ether exerts significant antifertility activity in female albino rats.[5]

Ripe fruit is alternative, digestive and antiscorbutic; and a cure for chronic diarrhoea. It is a rich source of Vitamin A, thiamine, riboflavine, niacin and ascorbic acid.[1]

Seeds of Carica papaya are black in colour and posses a cress-like odour. They are anthelmintic and carminative; and also considered to be powerful emmenagogue.[1] The seeds are shown to decrease fertility of albino mice but the activity has been conjectured for toxicity.[6]

Chemical analysis of finely powdered C. papaya seeds have shown to yield hentriacontane, β-sitosterol and free glucose.[7] Also the presence of benzylthiourea has been noted which has been held responsible for anthelmintic action.[7,8]

Although isolate concerning antifertility has not been traced so far, yet a recent observation by Prof. N.J. Chinoy and co-workers on C. papaya seed extract was quite interesting. They have shown that the aqueous extract of crushed seeds induces antifertility amongst male rats (personal communication). Accordingly it was decided to undertake collaborative work and initial studies on 70% ethanolic extract of seeds which exhibited antifertility activitywas undertaken.

PRESENT WORK

A 70% ethanolic aqueous extract of crushed papaya seeds was suspended in water and divided into fractions of upgraded polarity by successive partitioning with light petroleum ether, solvent ether, ethyl acetate and n-butanol (saturated with water) (Fig. 1). These fractions were individually tested for antifertility by oral administration in aqueous ethanol. Readings were noted in terms of sperm motility, total implantation sites and percentage fertility Table-1(A) .

However, efficacy data showed distribution of activity. Since subfractions were less active than that of the parent fraction, it was decided to locate the point of distribution by evaluating residual portions left after each solvent extraction Table-1 (B) . This led to two important observations.

1. Replicate experiments of lipophilic fractions often showed ununiformity in bioefficacy data.
2. Polar portion (solvent ether -R) was more efficacious (Table-1, B).

The former paradox could be partly removed by standardizing the method of administration in propylene glycol solution, which provided a better medium with respect to solubility. The latter result dictated further systematic chromatography of polar cuts of ethylacetate and n-butanol.

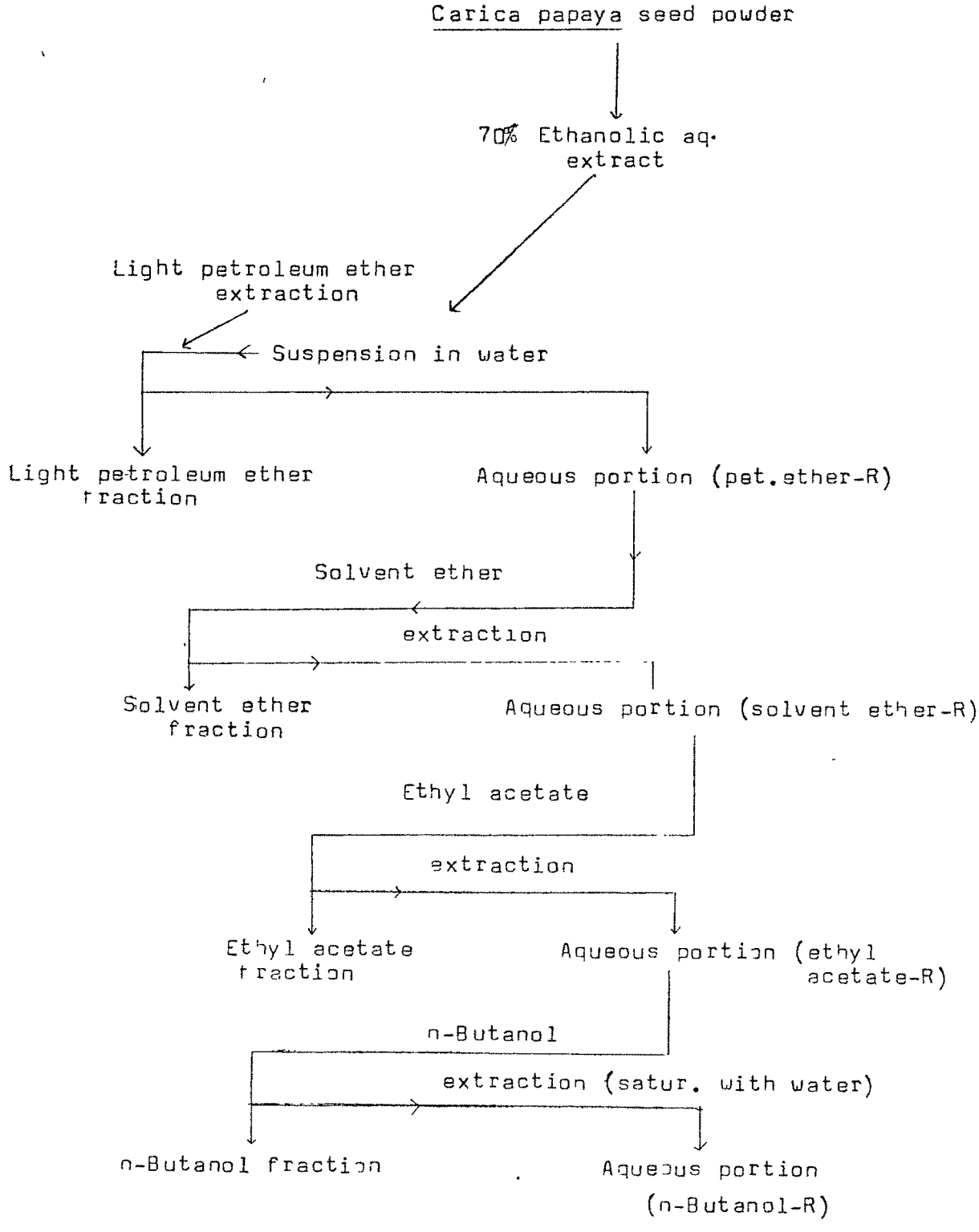

Fig. 1: Fractionation of 70% ethanolic extract of C. papaya seed powder

Table 1. Bioassay of 70% ethanolic extract of *C. papaya* seeds & its various fractions*

(A)

Entry	Fraction	Sperm motility	♀Pregnant/ ♀ Mated	Total number of implantation sites	Fertility rate(%)
1	Control	62.2	5/6	63.0	85.0
2	70% Ethanolic extract	19.9	0/6	0.0	0.0
3	Petroleum ether	10.35	2/4	13.0	50.0
4	Solvent ether	17.06	2/4	11.0	50.0
5	Ethyl acetate	9.56	1/4	11.0	25.0
6	n-Butanol	28.0	1/4	9.0	25.0

(B)

7	Pet ether-R	18.5	3/4	30.0	75.0
8	Solvent ether-R	16.38	0/4	0.0	0.0
9	Ethyl acetate-R	11.6	1/4	14.0	25.0
10	n-Butanol-R	9.6	1/4	9.0	25.0

*a) Dose - 5 mg/kg b.w., oral administration in propylene glycol
b) Results are averages of replicate experiments.

Ethyl acetate extract

Above extract when chromatographed on silica gel furnished methyl oleate and oleic acid (direct comparison with the authentic samples). p-Hydroxybenzoic acid could be isolated only after rechromatography of subfraction by flash elution; which was further subjected to bioassay. Replicate experiments showed variable results (Table 2.)

n-Butanol extract

The only isolable compound from this extract was ethyl glucoside ($\simeq 14\%$) and proved to be an artifact formed during initial ethanolic extraction. Aqueous extract of crushed seeds, however, was devoid of ethyl glucoside.

Out of the two possible anomers of ethyl glucoside, the one isolated was thermodynamically more stable β-anomer. This could be concluded by careful examination of ^{1}H-NMR spectrum ($CDCl_3$) of acetate derivative which showed subtle resonance peak of anomeric proton. The characteristic uofield shift (axial proton doublet, 4.52δ) and large value of spin-spin splitting (J = 8 Hz) due to anti-conformational relationship with vicinal proton made the assignment completely unambiguous. Moreover correlation with synthetically prepared β-ethyl glucoside as acetate derivative by CO-GLC corroborated the result (R_t : 4 min. 10% $DCQf_1$; column-200°C, FID-270°C).

Bioassay of ethyl glucoside showed reduction in sperm motility from 62.2 to 16.6 (Table-2). Therefore other three alkyl glucosides differing in carbon chain were prepared as the mixtures of α and β anomers[9] and tested (Table-2).

Methyl glucoside was found to be the most potent and caused further reduction in sperm motility to zero and hence exhibited no fertility. However, n-propyl glucoside had toxic effect. Dissection showed dead foetii in many replicates. Further increase in carbon chain had detrimental effect on activity and bioassay of n-butyl glucoside recorded 50% fertility.

In all the above cases dosage remained at 5 mg/kg body weight and could not be reduced further due to loss of activity at lower levels. This meant that some other minor constituent might have pronounced activity or might act synergistically with β-ethyl glucoside. With this aim in view, solvent ether extract was also rapidly analyzed.

Solvent ether extract

Column chromatography of solvent ether extract afforded benzylthiourea.[7,8] Efficacy data of this compound showed toxicity (Table 2). Number of foetii in treated animals were below normal and in some cases resorbed foetii were observed.

Table 2. Bioassay of isolates and synthetic glucosides

Entry	Compound	Sperm motility	♀Pregnant/ ♀ mated	Total number of implantation sites	Fertility rate(%)
1	p-Hydroxybenzoic acid	21.8,26.6, 31.4	2/6,0/6, 1/6	19,0,4	33,0,16.6
2	Benzylthiourea	18.5, 23.7	3/6,4/6	31*,44*	50,66
3	(α+β)-Ethyl glucoside	16.6	3/8	18	37.5
4	(α+β)-Methyl-glucoside	23.5	0/6	0	0
5	(α+β)-n-Propyl-glucoside	0,21	0/6,4/6	0,38*	0,66
6	(α+β)-n-Butyl-glucoside	26.4	3/6	34	50

* Foetii below normal or resorbed or ectopic.

However, it should be mentioned here that the results of biological systems in all the above cases were found to be variable. In order to resolve this discripancy many attempts like segregation of animals, improvement in administrative methods & changing strains of rats together with refined fractionations of extracts (counter current separation) proved futile. Thus it seems that bioassay needs further standardization and hence work has been discontinued till then.

EXPERIMENTAL

For General Methodology refer to Chapter-1.

Ethanolic extract of C. papaya seeds

Dry C. papaya seeds procured from Village Development Unit, P.O. Premnagar, Dist. Dehradun, Uttar Pradesh, India were powdered in pulverizer and sieved through 18 mesh sieve. Fine Powder (1.0 kg) was soaked in 70% ethanol in water (70 EtOH: 30 H_2O , 3 L) and after letting it stand at room temperature (30-35^{o}C) for 48 hr, the extract was withdrawn (900ml). The operation was repeated by adding appropriate quantity of fresh solvent as in Table 3. Extracts thus obtained were pooled together and freed of solvent to furnish 95.0 g of extract as a dark brown gummy mass.

Table 3. Extraction of C. papaya seeds

Extraction	Time hr.	Solvent recovered (ml)	Solvent added (ml)	Weight (g) of extract	Remarks
1	48	880	100	95.0	yield: 9.5% w.r.t. seed powder
2	64	700	100		
3	46	800	100		
4	72	1060	100		
5	72	980	100		

Partitioning of 70% ethanolic extract

Ethanolic extract (95 g) was suspended in demineralized water (450 ml) and then partitioned successively with light petroleum ether, solvent ether, ethyl acetate and n-butanol (saturated with water) as shown in Table 4. At the same time aliquots of the aqueous portion left after each solvent extraction were freed from water under reduced pressure on rotary evaporator (60°C/90 mm). Pet. ether-R, solvent ether-R, ethyl acetate-R and n-butanol-R, thus obtained (Table 1) were evaluated for bioefficacy together with organic solvent extracts.

Table 4. Solvent extraction of ethanolic extract

	Solvent	Volume (ml)	Weight of fractions (g)	Percentage by weight
1	Pet. ether	6 x 300	19.0	20.0
2	Solvent ether	6 x 300	0.75	0.8
3	Ethyl acetate	6 x 300	1.43	1.5
4	n-Butanol	5 x 300	6.2	6.5

Column chromatography of ethyl acetate extract

The ethyl acetate extract (5.1 g) was chromatographed on silica gel column (120 g, 66 x 2.2 cm) after adsorbing on silica gel (10 g) as follows:

Chromatogram -I

Frac.	Solvent system	Volume(ml)	Weight(g)	Remarks
1	Pet ether	3000	-	
2	20% Benzene in pet ether	3 x 250	0.3618	
3	-do-	17 x 250	0.3035	← Methyl oleate R_f: 0.5
4	40% -do-	16 x 250	0.0370	(5% EtOAc in pet ether)
5	60% -do-	6 x 250		
6	80% -do-	8 x 250	0.0024	
7	Benzene	2 x 250	0.0439	
8	Benzene	3 x 250	0.1346	← Oleic acid
9	Benzene	17 x 250	0.3531	R_f: 0.4 10% EtOAc/ pet ether
10	3% EtOAc in benzene	15 x 250	0.4001	
11	6% EtOAc in benzene	10 x 250	0.3434	
12	10% EtOAc in benzene	8 x 250		
13	30% EtOAc in benzene	4 x 250	0.5134	← p-OH-Benzoic acid
14	60% EtOAc in benzene	6 x 250	0.3824	(major)
15	80% EtOAc in benzene	6 x 250	0.2558	
16	Ethyl acetate	6 x 250	0.4800	
17	50% MeOH in ethyl acetate	4 x 250	1.4414	
			5.0528	Recovery

Analytical data of frac. 3 and Frc. 8 was identical with the authentic samples of methyl oleate and oleic acid in all respects. Fraction 13 (0.5 g) was rechromatographed on silica gel (20 g, 35.5 x 0.5 cm) by flash elution. Fraction was adsorbed on 0.5 g of silica gel and eluted at a flow rate of 25 ml/3 min as noted in Chromatogram-II.

Chromatogram II

Frac. (Pooled)	Solvent system	Volume (ml) of each frac.	Weight (g)	Remarks
1-2	Benzene	50	-	
3-4	4% EtOAc in benzene	50	-	
5-11	6% EtOAc in benzene	25	0.1048	Fairly pure
12-20	8% EtOAc in benzene	25		
21-27	9% EtOAc in benzne	25		
28-31	11% EtOAc in benzene	25	0.1696	p-Hydroxy-benzoic acid R_f: 0.25 60% EtOAc in benzene
32-47	11% EtOAc in benzene	25		
48-52	12% EtOAc in benzene	25		
53-55	15% EtOAc in benzene	25		
56-60	30% EtOAc inbenzne	25	0.2100	
			0.4844	Recovery

Isolated p-hydroxybenzoic acid was compared with the authentic sample by mixed melting point (212°C); ^{1}H-NMR and IR.

Column chromatography of n-butanol extract

Broad cut separation of n-butanol extract (5.72 g), adsorbed on 12 g silica gel, was carried out on silica gel column (115 g, 73 x 2.3 cm) by flash chromatography (flow rate-25 ml/min) as follows.

Chromatogram III

Frac.	Solvent system	Volume (L)	Weight(g)	Remarks
1	Ethyl acetate	2	0.6452	
2*	5% MeOH in EtOAc	2	1.7720	Rechromato-graphy
3	-do-	2	0.9622	
4	20% MeOH in EtOAc	4	1.3606	
5	-do-	1	0.1338	
6	50% MeOH in EtOAc	1	0.2250	
7	Ethanol	1	0.1447	
8	n-Butanol	1	0.0869	
			4.3304	Recovery

Fraction 2 (1.75 g) from the above Chromatography was rechromatographed on silica gel (34 g, 22.5 x 2.2 cm) as given in chromatogram IV.

Chromatogram IV

Frc.	Solvent system	Volume(ml)	Weight(g)	Remarks
1	Chloroform	100		
2	25% EtOAc in $CHCl_3$	250		
3	50% EtOAc in $CHCl_3$	250		
4	75% EtOAc in $CHCl_3$	250		
5	Ethyl acetate	2 x 100	0.0435	
6	Ethyl acetate	5 x 100	1.2101	
7	Ethyl acetate	10 x 100	0.2768	
8	Acetone	4 x 100	0.0331	
			1.5635	Recovery

Fraction 6 from the above chromatography was rechromatographed on silica gel to afford β-ethyl glucoside.

Chromatogram V

Wt. of fraction: 1.2 g, SiO_2-gel for adsorption: 1.5 g

SiO_2-gel for column bed: 19.0 g column dimensions: 32x 1.6 cm

Frac.	Solvent system	Vol (ml)	Wt.(g)	Remarks
1	50% EtOAc in pet ether	200	-	-
2	80% -do-	400	-	β-ethyl glucoside R_f: 0.35
3	-do-	5 x 100	0.7911	MeOH:EtOAc:$CHCl_3$
4	-do-	3 x 100	0.1121	
5	Ethyl acetate	100	-	5:5:10
			0.9032	Recovery

Acetylation of Fraction 3

TLC pure fraction (0.1049 g) was acetylated with pyridine (1 ml, distilled over KOH) and acetic anhydride (1 ml, freshly distilled). The reaction mixture was stirred at room temperature (30°C) for 14 hr. Excess reagents were stripped off at reduced pressure (60°C/20 mm), the product taken in ether (30 ml) washed thoroughly with water (3 x 10 ml) till neutral, then with brine (1 x 10 ml) and dried over anhydrous Na_2SO_4 to afford tetraacetate (0.1383 g). Solid thus obtained was crystallized in CCl_4 to furnish fine needles which melted at 108-109° (Lit.[10] m.p. 101-108°C), showed no depression in m.p. when mixed (1:1) with the authentic sample; and co-injected at R_t = 4 min. (10% DC_2F_1; Col. 200°, FID -270°).

Authentic sample for this purpose was prepared by acetylation of anomeric mixture of ethyl glucoside[9] followed by usual separation on silica-gel column.

R_f: β-Ethyl glucoside- 0.21 (30% EtOAc in pet. ether)

Preparation of alkyl glucosides (α + β, anomeric mixtures):

Methyl glucoside, ethyl glucoside, n-propyl glucoside and n-butyl glucoside were prepared as anomeric mixtures by Fischer's procedure as given in reference 9. Neutralization by silver carbonate/ion exchange and further purification by passage through silica column furnished pure glucosides with R_fs as given below.

Methyl glucoside : 0.34; Ethyl glucoside: 0.37

n-Propyl glucoside: 0.44; n-Butyl glucoside: 0.47.

Solvent system: $CHCl_3$: EtOAc: MeOH

10 : 5 : 5

Spray: 10% HNO_3 in conc. H_2SO_4.

Chromatography of solvent ether extract

Solvent ether extract (10.0 g) was chromatographed on silica gel (250 g, 76 x 3.3 cm) in petroleum ether with increasing percentage of ethylacetate.

Chromatogram VI

Frc.	Solvent system	Vol. collected	Weight (g)	Remarks
1-8	Pet. ether	8 x 200	-	
9-10	5% EtOAc in pet. ether	10 x 100	7.4724	
11	15% EtOAc in pet. ether	5 x 50		
*19-21	15% EtOAc in pet. ether	15 x 50	0.0300	←Benzylthiourea
22-29	-do-	8 x 250	0.4000	R_f: 0.66
30-37	30% -do-	8 x 250	1.000	(50% EtOAc in pet. ether)
38-42	50% -do-	5 x 250		
			8.9024	Recovery

Part of the pooled fraction (19-21) was recrystallized from 30% EtOAc in petroleum ether and then from chloroform to furnish

colourless crystals (m.p. 163°C). It was identified as benzylthiourea (Lit.[11]: m.p. 165°C).

BIOASSAY

All the bioassay evaluations incorporated in this Chapter were carried out at

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through the courtsy of Prof. N.J. Chinoy and co-workers.

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