In vitro evaluation of various crude extracts of seeds of *Trachyspermum Ammi (L.)* Sprague (Ajwain) for their potential inhibitory action on selected bacteria of clinical significance

Trachyspermum Ammi (L.) Sprague (Ajwaın) tohumlarından elde edilen çeşitli ham özütlerin klinik olarak önemli olan bazı bakterilere karşı potansiyel inhibisyon etkilerinin in vitro olarak test edilmesi

Research Article

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ABSTRACT

Seeds of *Trachyspermum ammi* (L.) Sprague (Ajwain) were evaluated for their antibacterial potential against several bacteria of clinical significance viz. *Staphylococcus aureus*, *Streptococcus pyogenes, Bacillus cereus, Bacillus subtilis, Escherichia coli* and *Salmonella typhi.* Extracts were found to inhibit one or more bacterial strains in different concentrations as observed by microbroth double dilution methodology. The pattern of inhibition depends largely on the solvent used for extraction and the organism tested. Extracts prepared in organic solvents were found more active than aqueous extracts. Furthermore, methanol extract was found to have greater activity against almost all the bacterial species tested. Gram positive bacteria were found more sensitive when compared to Gram negative bacteria. *Staphylococcus aureus* was found to be most susceptible followed by *Bacillus* species. *Salmonella typhi* and *Streptococcus pyogenes* were found to be most resistant bacteria, however, *Escherichia coli* showed mild sensitivity to some of the extracts. The study reveals the possibility of the presence of antibacterial components in the Ajwain seeds, thus it can assure an interesting future prospect in the world of medicine for the discovery of novel agents with antimicrobial potential.

Key words

Trachyspermum ammi, Ajwain, Antibacterial activity, Agar-well diffusion assay, MIC.

ÖZET

Trachyspermum ammi (L.) Sprague (Ajwain) tohumlarının antibakteriyal potansiyelleri; klinik olarak önemli *Staphylococcus aureus*, *Streptococcus pyogenes*, *Bacillus cereus*, *Bacillus subtilis*, *Escherichia coli* ve *Salmonella typhi* bakterilerine karşı değerlendirilmiştir. Mikrobroth çift seyreltme yönteminde belirlenen şekilde farklı derişimlerde hazırlanan özütlerin bir ya da daha fazla bakteriyel suşunu inhibe ettiği bulunmuştur. İnhibisyon modeli büyük ölçüde özütleme sırasında kullanılan çözücüye ve test edilen organizmaya bağlıdır. Organik çözücülerde hazırlanan özütlerin sulu çözeltilerde hazırlananlara göre daha etkin olduğu bulunmuştur. Ayrıca, metanolün çözücü olarak kullanıldığı özütlerin hemen hemen tüm bakteri türlerine karşı çok daha büyük bir inhibisyon etkisine sahip olduğu bulunmuştur. Gram pozitif bakterilerin, gram negatif bakterilerden daha duyarlı olduğu bulunmuştur. *Staphylococcus aureus*'un Bacillus türlerinden sonra en duyarlı suş olduğu bulunmuştur. *Salmonella typhi* ve *Streptococcus pyogenes* suşlarının en dirençli bakteriler olduğu belirlenmiştir, *Escherichia coli*'nin ise bazı özütlere karşı hafif bir duyarlılık gösterdiği tespit edilmiştir. Bu çalışma Ajwain tohumlarında antibakteriyel bileşenlerin var olduğunu ortaya çıkarmıştır, bu nedenle tıp dünyası için gelecekte antimikrobiyal potansiyale sahip ham maddelerin keşfi adına umut vaat ettiği söylenebilir

Anahtar Sözcükler

Trachyspermum ammi, Ajwain, Antibakteriyel aktivite, Oyuk agar difüzyon yöntemi, MIC

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INTRODUCTION

Antibiotic chemotherapy has been one of the most important medical achievements of the twentieth century. The subsequent development of antibiotics has produced impressive reductions in the burden of diseases imposed by microbial infections [1,2]. The increase of antibiotic resistance among microorganisms to conventional drugs has necessitated the search for new, efficient and cost effective ways for the control of infectious diseases. Many studies have showed that medicinal plants constitute a great source for the isolation of active drugs, e.g. emetine, which has been used for a long time for the treatment of amebiasis and other diseases, was isolated from plant as well as quinine used for the treatment of malaria [3].

The subject of medicinal plants is a highly active field of scientific study all over the world. Medicinal plants are an important part of human history, culture and tradition. Several centuries ago, lime fruits were given to British seamen who miraculously recovered from the symptoms of scurvy. It took science about 200 years to find out why (the discovery of vitamin C). It is likely that some traditional medicinal plants hold the key to new advances of great importance to human health [4].

Plants have been referred as the basic source for medicines with earliest references in the *Vedas*; the most ancient literature on the earth. The *Rigveda* is oldest among all the *Vedas* and its X^{th} Mandal has been given in the praise of medicinal plants. The *Atharvaveda* is considered to be another important *Veda*, which is believed to be the mother of Ayurveda, the science of life. It describes about 129 medicinal plants, however, some claim 280. The *Yajurveda* and *Samveda* are known to be full of information about the medicinal plants. Medicinal knowledge of plants has also been described in various Samhitas and Nighantus which are believed to be derived from the Vedas later on [5]. The advantage of using higher plants is that a sustainable supply of desired metabolites can be achieved, which is not always possible for a microbial product; moreover, higher plants promise greater opportunities with all relatively cheap and easy approaches against some expensive and technically complex methods presently in use for new drug discovery [3]. It involves a multidisciplinary approach combining botanical, ethnobotanical, phytochemical and biological techniques.

Anecdotal evidence and the traditional use of plants as medicine provide the basis for indicating which essential oil(s) and plant extract(s) may be useful for specific medical conditions. Historically, many plant extracts and essential oils, such as tea tree, myrrh and clove, have been used as topical antiseptics, or have been reported to have antimicrobial properties [6,7]. The antimicrobial properties of essential oils derived from many plants have been empirically recognized for centuries, but scientifically confirmed only recently [8].

Therefore, current study was carried out to evaluate the antibacterial potential of *Trachyspermum* ammi (L.) Sprague (Ajwain) seeds under *in vitro* conditions. Ajwain (Sanskrit: *Yavanaka* or *Yavani*) is an important spice having a number of therapeutic properties and it belongs to family Apiaceae (Parsley family). Mainly, it helps against diseases of the digestive tract and fever. Thymol is the main ingredient of seeds and is used in medicines against cough and throat irritation. Furthermore, α-pinene, *p*-cymene, limonene and γ-terpinene have also been found in seed extracts.

MATERIAL AND METHODS

Extraction of plant material: Ajwain seeds were collected locally. Seeds were washed thoroughly first under running tap water followed by sterilized distilled water. They were then air-dried and powdered with the help of sterilized pestle and mortar. The powder was further extracted by using aqueous and organic solvents as given below:

Aqueous extraction: 10 g of air-dried powder was boiled in 400 ml distilled water till one fourth of the extract initially taken was left behind after

evaporation. The solution was then filtered using muslin cloth. Filtrate was centrifuged at 5000 rpm for 15 minutes. The supernatant was again filtered using Whatman filter no. 1 under strict aseptic conditions and the filtrate was collected in fresh sterilized bottles and stored at 4°C until further use.

Organic solvent extraction

10 g of air-dried powder was thoroughly mixed with 100 ml organic solvent (*viz*., Ethanol, Methanol, Hexane and Ethyl acetate). The mixture was placed at room temperature for 24 h on shaker with 150 rpm. Solution was filtered through muslin cloth and then re-filtered by passing through Whatman filter no.1. The filtrate thus obtained was concentrated by complete evaporation of solvent at room temperature to yield the pure extract. Stock solutions of crude extracts for each type of organic solvent were prepared by mixing well the appropriate amount of dried extracts with respective solvent to obtain a final concentration of 100 mg/ml. Each solution was stored at 4°C after collecting in sterilized bottles until further use.

Bacterial Strains

The bacterial strains selected for present study were collected from Microbial Type Culture Collection (MTCC). A total of six bacteria namely *Escherichia coli* and *Salmonella typhi* (both are Gram-negative bacteria) and *Bacillus cereus*, *Bacillus subtilis*, *Staphylococcus aureus* and *Streptococcus pyogenes* (all Gram-positive bacteria) were screened for present investigation. These bacterial cultures were maintained in nutrient agar slants at 37°C. Each of the microorganisms was reactivated prior to susceptibility testing by transferring them into a separate test tube containing nutrient broth and incubated overnight at 37°C.

Antibacterial Assay: *In vitro* antibacterial activity of all aqueous and organic extracts of Ajwain seeds was determined by standard agar well diffusion assay assay [10]. Petri dishes (100 mm) containing 25 ml of Mueller Hinton Agar (MHA) seeded with 100 µl inoculum of bacterial strain (inoculum size was adjusted so as to deliver a final inoculum of approximately 10^8 CFU/ml). Media was allowed to solidify and then individual Petri dishes were marked for the bacteria inoculated. Wells of 6 mm diameter were cut into solidified agar media with the help of sterilized cup-borer. 100 µl of each extract was poured in the respective well and the plates were incubated at 37°C for overnight. Organic solvents were used as negative control while tetracycline (5 µg ml^{-1}) was used as a positive control. The experiment was performed in triplicate under strict aseptic conditions and the antibacterial activity of each extract was expressed in terms of the mean of diameter of zone of inhibition (in mm) produced by the respective extract at the end of incubation period.

Determination of Minimum Inhibitory Concentration

Active extracts thus obtained were screened to determine minimum inhibitory concentrations (MICs) by standard two-fold microbroth dilution methodology given by NCCLS [9]. A stock solution of each active extract was serially diluted in 96-wells microtiter plate with Mueller Hinton broth to obtain a concentration ranging from 8 µg/ml to 4096 µg/ml. A standardized inoculum for each bacterial strain was prepared so as to give inoculum size of approximately 5 x 10^5 CFU/ml in each well. Microtiter plates were then kept at 37°C for an overnight incubation. Following incubation, the MIC was calculated as the lowest concentration of the extract inhibiting the visible growth of bacterial strain. All the chemical ingredients used in present study were of analytical grade, and were purchased from Hi Media, India.

RESULTS AND DISCUSSION

Results (as shown in Table 1) indicated that methanol extract was found to have maximum inhibitory power with zones of inhibition ranges from 11.14 mm to 20.67 mm with maximum inhibition of *Staphylococcus aureus*, moderate inhibition of *Escherichia coli*, *Bacillus cereus* and *Bacillus subtilis* and mild inhibition of *Streptococcus pyogenes* and *Salmonella typhi*.

Ethanol extract was comparatively lower inhibitory than that of methanol extract. This extract was found active against only four out of six bacteria evaluated. Ethyl acetate and aqueous extracts were found to be moderate effective against three and two bacteria, respectively. Hexane extract was shown to have very mild activity only against *Staphylococcus aureus*. The maximum susceptibility was shown by *Staphylococcus aureus* which was inhibited by all the ajwain seed extracts followed by *Bacillus subtilis* and *Bacillus cereus* (inhibited by methanol, ethanol and ethyl acetate extracts), *Escherichia coli* (inhibited by ethanol, methanol and aqueous extracts). *Streptococcus pyogenes* and *Salmonella typhi* were shown to have maximum resistance against evaluated crude extracts and were inhibited only by methanol extract. Antibacterial activity in terms of inhibition zones produced by various crude extracts of Ajwain against different microflora has been shown in Figure 1.

Active crude extracts thus obtained were evaluated for minimum inhibitory concentrations

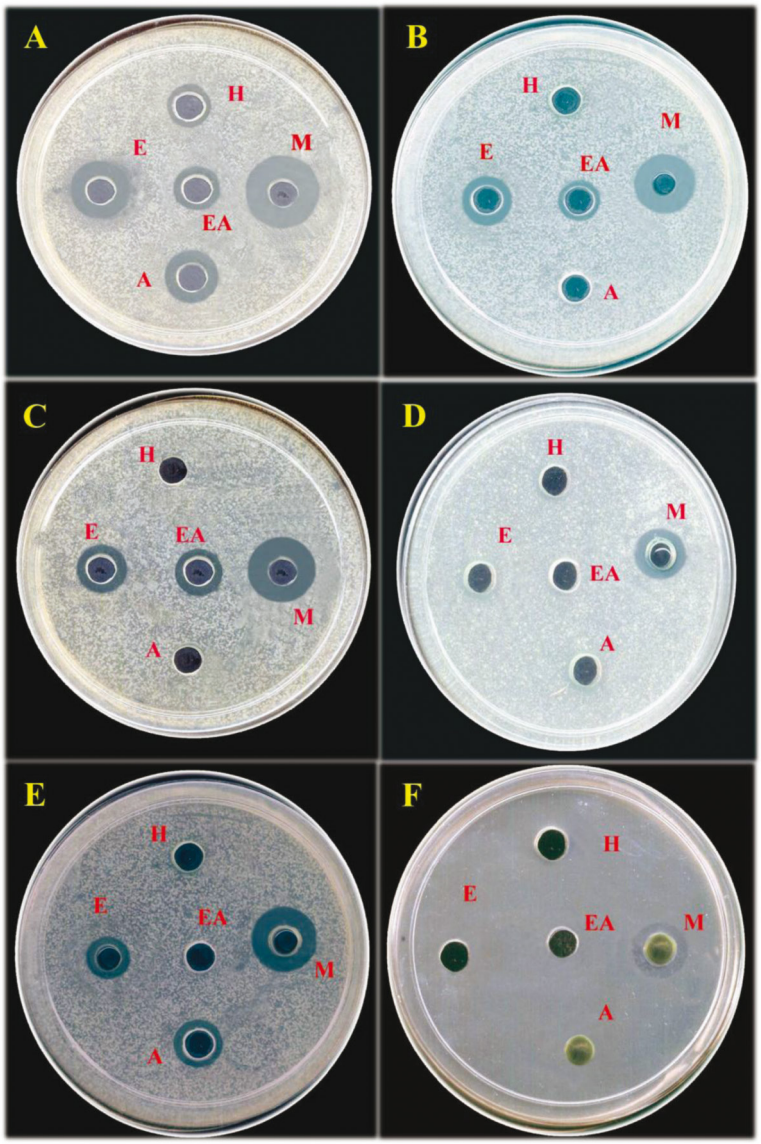

Figure 1. Inhibition Zone(s) Exhibited by various crude extracts of *Trachyspermum ammi* (L.) Spraque (Ajwain) Seeds against; (A) *Staphylococcus aureus*; (B) *Bacillus subtilis*; (C) *Bacillus cereus*; (D) *Streptococcus pyogenes*; (E)*Escherrihia coli* and (F) *Salmonella typhi*.
Indications:H-Hexane extract (upper well); A-Aqueous extract (lower well); E-Ethanol extract (left well); M-Methanol extract (right well); EA-Ethyl Acetate extract (central well).

(MICs) against respective susceptible bacteria (Table 2). Methanol extract was found significant inhibitory against *Staphylococcus aureus* with MIC 256 μg ml^{-1}. The same extract was found active against *Bacillus cereus* and *Escherichia coli* at 2048 μg ml^{-1} concentration (Figure 2). MIC of this extract against *Bacillus subtilis* was observed as 4096 μg ml^{-1}. *Staphylococcus aureus* was also inhibited by other extracts *viz*., ethanol and aqueous at the concentration of 1024 μg ml^{-1} and 4096 μg ml^{-1}, respectively; while the aqueous extract didn't show the inhibition of *Escherichia coli* in the entire range of extract's dilutions (*i.e.* >4096 μg ml^{-1}).

Tetracycline was used as a standard antibiotic throughout the study for a comparative analysis of antibacterial activity of various crude seed extracts of *Trachyspermum* ammi (L.) Sprague (Ajwain).

The tested extracts of *Trachyspermum* ammi (L.) Sprague (Ajwain) Seeds appear to be effective against a wide spectrum of microorganisms including both Gram-positive and Gram-negative bacteria; they may be able to control a wide range of microbes including pathogens. While many variables beyond the scope of this study exist, it is safe to conclude that the plant investigated is indeed effective as antibacterial agents. A detailed biochemical analysis such as GC-MS, FT-IR and NMR could quantitatively define the antimicrobial effectiveness of medicinal plants by identifying a specific compound(s) responsible for such properties.

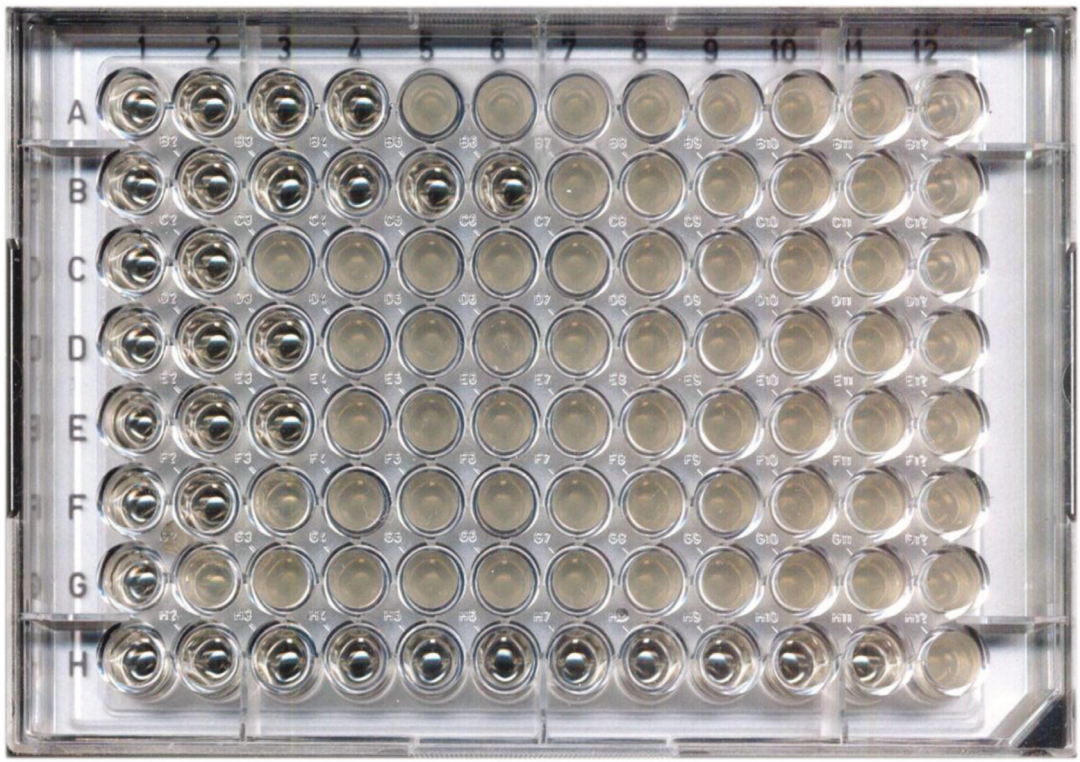

Figure 2. Minimum Inhibitory Concentration(s) of Active Crude Extracts of Trachyspermum ammi (L.) Spragne (Ajwain) Seeds: Picture represents a 96-Well Microtiter Plate showing the Inhibition of Microbial Growth;

Row: A) Ethanol extract against *Staphylococcus aureus*; (B) Methanol extract against *Staphylococcus aureus*; (C) Methanol extract against *Bacillus subtilis*; (D) Methanol extract against *Bacillus cereus*; (E) Methanol extract against *Escherrihia coli* and (F) Aqueous extract against *Staphylococcus aureus*; (G) Aqueous extract against *Escherrihia coli*; (H) Tetracycline (standard antibiotic) against *Staphylococcus aureus*.

Colomns:

1: Negative control (CAMHB)

2-11: Extracts dilution 4096 μg/ml to 8 μg/ml,

12: Positive control (CAMHB + Respective Microbial Culture).

Table 1. In Vitro Antibacterial Activity of Aqueous and Organic Extracts of Trachyspermum ammi (L.) Sprague (Ajwain) Seeds,

Type of Extract			Zone of Inhibition* (in mm diameter)					
			Gram-negative Bacteria		Gram-positive Bacteria			
			Escherichia coli	*Salmonella typhi*	*Streptococcus pyogenes*	*Staphylococcus aureus*	*Bacillus subtilis*	*Bacillus cereus*
Organic Extract	Ethanol		10.21±1.06	NI	NI	15.50±0.86	11.02±0.17	10.83±0.58
	Methanol		15.03±0.71	11.14±0.17	11.43±0.12	20.67±0.23	14.66±1.23	15.10±1.10
	Ethyl Acetate		NI	NI	NI	12.05±0.73	10.27±1.52	10.16±0.76
	Hexane		NI	NI	NI	10.23±0.58	NI	NI
Aqueous Extract			12.34±0.24	NI	NI	14.16±1.54	NI	NI
Control	**Positive**	Tetracycline⁺	29.50±0.50	25.83±1.61	29.83±1.89	32.50±1.50	34.17±1.76	32.16±1.04
	Negative	Ethanol	NI	NI	NI	NI	NI	NI
		Methanol	NI	NI	NI	NI	NI	NI
		Ethyl Acetate	NI	NI	NI	NI	NI	NI
		Hexane	NI	NI	NI	NI	NI	NI

Table 2. Minimum Inhibitory Concentration of Active Crude Extracts of Trachyspermum ammi (L.) Sprague (Ajwain) Seeds.

Type of Active Crude Extract	Test Microorganism	Concentration of Extracts* (in μg ml^{-1})										MIC (in μg ml^{-1})
		4096	2048	1024	512	256	128	64	32	16	8	
Ethanol	*Staphylococcus aureus*	-	-	-	+	+	+	+	+	+	+	1024
Methanol	*Staphylococcus aureus*	-	-	-	-	-	+	+	+	+	+	256
Methanol	*Bacillus subtilis*	-	+	+	+	+	+	+	+	+	+	4096
Methanol	*Bacillus cereus*	-	-	+	+	+	+	+	+	+	+	2048
Methanol	*Escherichia coli*	-	-	+	+	+	+	+	+	+	+	2048
Aqueous	*Staphylococcus aureus*	-	+	+	+	+	+	+	+	+	+	4096
Aqueous	*Escherichia coli*	+	+	+	+	+	+	+	+	+	+	>4096
Tetracycline	*Staphylococcus aureus*	-	-	-	-	-	-	-	-	-	-	<8

*Different concentrations of active crude extracts evaluated in 96-well microtiter plate using Microbroth Dilution Assay as recommended by NCCLS. All values are expressed in μg ml^{-1}; (-) represents 'No Growth Observed'; (+) represents 'Growth Observed'

*Values of the observed zone of inhibition (in mm diameter) including the diameter of well (6 mm) after 24 hours incubation against different bacterial species when subjected to different extracts in agar well diffusion assay. Assay was performed in triplicate and results are the mean of three values ± Standard Deviation. In each well, the sample size was 100 μl. Inhibition observed in extracts due to solvent were assessed through negative controls. 'NI'-No Inhibition Zone was observed. *Tetracycline (5 μg ml^{-1}) was used as standard antibiotic.

REFERENCES

1. I. Chopra, J. Hodgson, B. Metcalf, and G. Poste, The search for antimicrobial agents effective against bacteria resistant to multiple antibiotics. Antimicrobial Agents and Chemotherapy, 41 (1997) 497.
2. D.J.C. Knowles, New strategies for antibacterial drug design. Trends in Microbiology, 5 (1997) 379.
3. M.M. Cowan, Plant products as antimicrobial agents. Clinical Microbiology Review, 12 (1999) 564.
4. B.E. Wyk, and M. Wink, Medicinal Plants of the World, Ist Edition. Briza publications, Arcadia 0007, Pretoria, South Africa, (2004) 1-480.
5. P. Kaushik, Glimpses of Medical Botany in Atharvaveda (Kand IV). The Vedic Path, 48 (1985) 64.
6. M. Lis-Balchin, and S.G. Deans, Bioactivity of selected plant essential oils against Listeria monocytogenes. Journal of Applied Microbiology, 82 (1997) 759.
7. K.A. Hammer, C.F. Carson, and T.V. Riley, Antimicrobial activity of essential oils and other plant extracts. Journal of Applied Microbiology, 86 (1999) 985.
8. S.G. Deans and G. Ritchie, Antibacterial properties of plant essential oils. International Journal of Food Microbiology, 5 (1987) 165.
9. NCCLS-National Committee for Clinical Laboratory Standards. Methods for Dilution Antimicrobial Susceptibility Tests for Bacteria that Grow Aerobically, Approved Standards M7-A4, Wayne, Pa. 1997.
10. C. Perez, M. Pauli, P. Bazerque, An antibiotic assay by the agar-well diffusion method. Acta Biologiae et Medecine Experimentalis, 15 (1990) 113-115.