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Phytochemical screening of cryo-grinded Ajwain (*Trachyspermum ammi* L.)

L.K. Sharma[1], D. Agarwal[1], Sapna Jain[2], S. John[1], R. Kumar[1], Amit Bhargav[3], M.K. Mahatma*[1]& S.N. Saxena[1]

[1]ICAR-National Research Centre on Seed Spices, Ajmer, Rajasthan, India
[2]Maharishi Dayanand University, Ajmer, Rajasthan, India
[3]Bhupal Nobles' University, Udaipur, Rajasthan, India

Abstract

Ajwain is a rich source of phytochemicals. To find out best solvent for phytochemical extraction various solvents i.e. water, alcohol, hydro-alcoholic (70:30), benzene, petroleum ether, hexane and methanol were used. Maximum extractive value was observed with hexane (16.28) followed by alcohol (16.04) and methanol (14.75) solvent. Further phytochemicals analysis were carried out using hexan and methanol solvent.Qualitative chemical tests were conducted to identify various phytoconstituents in all samples. Various tests were performed to identify carbohydrates, proteins, steroids, amino acids, flavonoids, alkaloids, tennis, vitamin, glycosides and triterpenoids in ajwain. In ajwain carbohydrate, protein, amino acid, steroids, and flavonoids were detected in both the solvent and all the tests were positive. Alkaloids, Triterpenoids and vitamin tests were absent in both the solvent of ajwain. Tenins and glycosides were present in some test of methanol extract and also hexane extract of ajwain. It is considerably easier and cheaper to perform a preliminary phytochemical screening by using qualitative methods.

Key words: Ajwain, amino acid, cryo-grinding, carbohydrate, hexane, methanol, protein.

Introduction

Ajwain (*Trachyspermum ammi* L.) is a small, annual, herbaceous plant with erect,branched leafy stems, feather-like leaves (2.5 cm long), and 4-12 ray flower heads bearing 6 - 16 flowers. The fruits are minute, greyish-brown coloured and egg-shaped. Cultivation of this plant originated in Egypt. It grows widely around the Mediterranean Sea and in South-West Asia extending from Iraq to India, particularly North India- Madhya Pradesh, Gujarat, Maharashtra, Uttar Pradesh, Punjab, Haryana, Rajasthan, Bihar and West Bengal. Ajwain is frequently used as a spice in curries due to its fragrance perfume and spicy flavour. Its seeds are used in little amounts to flavor various meals, as preservatives, in medicine, and to make essential oils for use in perfumery (Pruthi, 1992).

It is utilised as a home cure for stomach problems in India, where a paste of crushed fruits is used externally to relieve colic symptoms and a hot and dry fermentation of the fruits applied to the chest is used as a typical asthma therapy (Anonymous, 1995). Researchers have discovered anti-aggregatory, anthelmintic, anti-hyperlipidaemic, antifilarial, insecticidal, kidney stone inhibitory, molluscicidal, mosquito repellant, and nematicidal properties in ajwain. The essential oil of seeds also exhibited toxicity against *Epidermophyton floccsum, Microsporum canis and Trichophyton mentagrophytes* fungus. Fungitoxicity of the oil was thermostable up to 150,°C and thymol was identified as the fungitoxic chemical in essential oil (Singh *et al.,* 1986). The seed extract was reported to possess fungicidal activity against *Aspergillus clavatus Candida albicans Rhizoctonia solani*, which causes rice sheath blight (Ansari, 1995, Sharma *et al.,* 2018). Antibacterial activities of ajwain oil were observed against *Staphylococcus aureus, Escherichia coli, Salmonella typhi, Shigelladysenteriae* and *Vibrio cholera* (Syed *et al.,* 1986; Anonymous, 1995). The essential oils extracted from ajwain seeds showed anti-bacterial activity (Mayaud *et al.,* 2008; Singh *et al.,* 2002). Extracts made in various solvents have varying levels of efficacy against *E. coli, Pseudomonas aeruginosa, Salmonella typhi*, and *Staphylococus. aureus* (Ahmad *et al*., 1998; Patel *et al.,* 2008, Sharma *et al.,* 2018), suggesting that they have been used in the treatment of gastrointestinal problems for centuries. Kaur and Arora (2009) discovered that aqueous and organic extracts of ajwain seeds had antibacterial properties, confirming the ancient use of ajwain seeds to treat a variety of gastrointestinal ailments.

*Corresponding author email: maheshmahatma@gmail.com

This historical use of ajwain seeds to cure various gastrointestinal disorders has also been scientifically proved in another study carried out by Kaur and Arora (2009) wherein aqueous and organic extracts of ajwain seeds have also shown their antibacterial effect. Methanol extracts of ajwain seed demonstrated a robust *in-vitro* inhibitory effect on hepatitis C virus (HCV) protease at a dose of 100 gmL^{-1} (Hussein *et al.,* 2000). Moreover, the methanol extract of Ajwain seeds possesses anti-oxidative properties (Saxena *et al.,* 2012).The type of solvent that is used is the primary factor impacting the extraction of phytochemicals from plant material.Therefore, the present investigation aimed to find out the best solvent to get maximum extractive value and to detect phytochemicals present in ajwain seeds.

Materials and methods

Seeds of ajwain (variety AA-2) were obtained from the seed store of ICAR-NRCSS, Ajmer for the present study.

Grinding of seeds

Grinding of seeds was done using a cryogenic grinder (Spectra Cryogenics, Rajasthan, India). Feed rate of material was set at 1 kg hr^{-1} with a screw speed of 3 rpm. Inlet temperature was adjusted to below -30 ^{0}C and outlet temperature was -5 to 15 ^{0}C. Product particle size was set at 50 microns. The cryo ground powder was quickly packed in aluminium foil packets using a sealing machine and opened at the time of analysis.

Physico-chemical screening of Ajwain powder

The cryogenically ground seed extract of ajwain was used for phytochemical screening of seed extract

a. Determination of alcohol-soluble extract: Air-dried Ajwain powder (5g) was macerated with 100 mL of absolute ethanol in a closed flask for six hours with frequent shaking and allowed to stand for 18 h. Homogenized was filtered rapidly taking precautions against loss of ethanol. Evaporated to dryness in a tared flat bottom shallow dish, dried at 105±1 ^{0}C and weighed. Calculated the percentage of alcohol-soluble extractive with reference to the air-dried drug.

b. Determination of water-soluble extract: Air-dried Ajwain powder (5g) was macerated with 100 mL of water in a closed flask for six hours with frequent shaking and allowed to stand for 18 h. Homogenized was filtered rapidly taking precautions against loss of water. Evaporated to dryness in a tared flat bottom shallow dish, dried at 105±1^{0}C and weighed. Calculated the percentage of water-soluble extractive with reference to the air-dried drug.

c. Determination of Benzene soluble extract: Air-dried Ajwain powder (5g) was macerated with 100 mL of benzene of the specified strength in a closed flask for six hours with frequent shaking and allowed to stand for 18 h. Homogenized was filtered rapidly taking precautions against loss of extract. Evaporated to dryness in a tared flat bottom shallow dish, dried at 105±1^{0}C and weighed. Calculated the percentage of water-soluble extractive with reference to the air-dried drug.

d. Determination of Petroleum ether extract: Air-dried Ajwain powder (5g) was macerated with 100 mL of petroleum ether of the specified strength in a closed flask for six hours with frequent shaking and allowed to stand for 18 h. Homogenized was filtered rapidly taking precautions against loss of extract. Evaporated to dryness in a tared flat bottom shallow 7 dishes, dried at 105±1^{0}C and weighed. Calculated the percentage of water-soluble extractive with reference to the air-dried drug.

e. Determination of Hydro-alcohol extract: Air-dried Ajwain powder (5g) was macerated with a mixture of 70 mL of ethanol and 30 mL water in a closed flask for six hours with frequent shaking and allowed to stand for 18 h. Homogenized wasfiltered rapidly taking precautions against loss of ethanol. Evaporated to dryness in a tared flat bottom shallow dish, dried at 105±1^{0}C and weighed. Calculated the percentage of alcohol-soluble extractive with reference to the air-dried drug.

f. Determination of Hexane extract: Air-dried Ajwain powder (5g) was macerated with 100 mL of hexane in a closed flask for six hours with frequent shaking and allowed to stand for 18 h. Homogenized was filtered rapidly taking precautions against loss of hexane. Evaporated to dryness in a tared flat bottom shallow dish, dried at 105±1^{0}C and weighed. Calculated the percentage of water-soluble extractive with reference to the air-dried drug.

g. Loss on drying: Ajwain powder (2 g) was accurately weighed in a china dish and kept in a hot air oven maintained at 110±1^{0}C for four hours. After cooling in a desiccator, the loss in weight was recorded. This procedure was repeated till constant weight was obtained.

h. Determination of ash value: Ajwain powder (2 g) was taken in a pre-weighted silica crucible and kept in a muffle furnace for 6 h at 600^{0}C. Ashed sample crucibles were transferred to a desiccator and cool to room

temperature. After cooling crucibles were weighed as quickly as possible to prevent moisture absorption. Total ash % was calculated as: % ash = weight of residue x 100/ weight of the sample.

i. Determination of acid-insoluble ash: Ash was boiled for 5-10 minutes with 25 mL of diluted hydrochloric acid. After that insoluble matter was collected in a Gooch crucible, washed with hot water, and then ignited and weighed. Calculated the percentage of acid insoluble ash with reference to air-dried drugs. Acid insoluble ash value of the sample % = Where, a = weight of the residue y = weight of air-dried drug

j. Determination of water-soluble ash: Ash was boiled for 5-10 minutes with 25 mL of water, After that insoluble matter was collected in a Gooch crucible, washed with hot water and ignited to constant weight at a low temperature. Subtracted the weight of insoluble matter from the weight of ash. The difference in weight represents the water-soluble ash. Calculated the percentage of water-soluble ash with reference to the air-dried drug. Water-soluble ash- Weight of insoluble matter Percentage of water-soluble ash = Where, a = water-soluble ash b = air-dried drug.

Preliminary phytochemical

Investigation of ajwain extracts Qualitative chemical tests were conducted to identify the various phytoconstituents present in samples. The various tests and reagents used are given below.

1. Tests for Carbohydrates: Molisch's test (general test): Test solution (2 mL) was taken in a test tube and a few drops of ά-naphthol solution (prepared in alcohol) were added. Test tubes were shaken and (quantity) concentrated H_2SO_4 added from the sides of the test tube, a violet ring was observed at the junction of two liquids.

For Reducing sugars: - a) Fehling's test: 1 mL Fehling's A and 1 mL Fehling's B solution were mixed and boiled for one minute. Added equal volume of T.S. Heated in boiling water bath for 5-10 min was observed for a yellow, then brick red precipitate. b) Benedict's test: Equal volume of benedict's reagent and T.S. in the test tube were mixed. Heated in boiling water bath for 5 min. The solution may appear green, yellow or red depending on the amount of reducing sugar present in T.S. Tests for Monosaccharides Benedict's test: Equal volume of Barfoed's reagent and T.S. were added. Heated for 1-2 min, in boiling water bath and cooled. Observed for red precipitate. Tests for Hexose's sugar Cobalt-chloride test: 3 mL of T.S. was mixed with 2 mL cobalt chloride, boiled and cooled. Added $FeCl_3$ drops on NaOH solution. Solution observed for greenish-blue (glucose), purplish (Fructose) or upper layer greenish-blue and lower layer purplish (Mixture of glucose and fructose). Tests for Non-Reducing Sugars a) Test sample did not give response of Fehling's and Benedict's test. b) Tannic acid test for starch: With 20% tannic acid, test solution was observed for precipitate.

2. Tests for Proteins: a) Biuret test (General test): Known quantity of test solution (1 mL) was mixed with 3 mL of biuret reagent (4% NaOH and few drops of 1% $CuSO_4$ solution observed for violet or pink colour. b) Million's test (for proteins): Mixed 3 mL test solution with 5 mL Million's reagent, white precipitate obtained. Precipitate warmed turns brick red or precipitate dissolves giving red color was observed. c) Xanthoprotein test (For protein-containing tyrosine or tryptophan): Mixed 3 mL test solution with 1 mL concentrated H_2SO_4 observed for white precipitate. d) Test for protein-containing Sulphur: Mixed 5mLtest solution with 2 mL 40% NaOH and 2 drops 10% lead acetate solution. The solution was boiled it turned black or brownish due to PbS formation was observed. e) Precipitation test: The test solution gave white colloidal precipitate with the following regents: i) Absolute alcohol ii) 5% mercuric chloride solution iii) 5% cupric sulphate solution iv) 5% lead acetate v) 5% ammonium sulphate

3. Tests for Steroid : a) Salkowski Reaction: To 2 mL of test solution, 2 mL chloroform and 2 mL concentrated H_2SO_4 were added. The mixture was shaken well, after that chloroform layer appeared red and acid layer showed greenish-yellow fluorescence was observed. b) Libermann - Burchard Reaction: Mixed 2 mL test solution with chloroform. Acetic acid (1-2 mL) and 2 drops of concentrated H_2SO_4were added from the side of test tube, initially red colour appeared, then blue and finally green color appeared. c) Libermann's reaction: Test solution (3 mL) was mixed with 3 mL acetic anhydride. Mixture was heated and cooled. Few drops of concentrated H_2SO_4 were added and observed for blue colour.

4. Tests for Amino acids: a) Ninhydrin test (General test): - Three drops of 5% Ninhydrin solution were mixed with test solution (3 mL) and heated in boiling water bath for 10 min. Observed for purple or bluish colour. b) Test for tyrosine: Three drops of Million's reagent added in 3 mL test solution and heated. Solution observed for dark red colour. c) Test for tryptophan: Few drops of glycoxalic acid and concentrated H_2SO_4 were added in a 3 mL test

solution, observed for a reddish violet ring at the junction of the two layers.

5. Test for flavonoids: a) Shinoda test: To test solution 5 mLof 95% ethanol was added, then few drops of concentrated HCl and 0.5g magnesium turning were mixed. The pink colour was observed. b) To small quantity of residue, ead acetate solution was added, observed for yellow-coloured precipitate. c) Addition of few drops of dilute NaOH with test solution showed yellow colouration, which was decolorized after the addition of acid was observed. d) Ferric chloride test: Few drops of ferric chloride solution were added in a test solution, and an intense green colour was observed.

6. Test for Alkaloids: a) Dragendrff's test: To 2-3 mL test solutionfew drops of Dragendroff's regent were added and observed for orange-brown precipitate. b) Mayer's test: Few drops of Mayer's reagent were added in 2-3 mLof test solution and then observed for precipitate. c) Hager's test: Few drops of Hager's reagent were mixed with 2-3 mL of test solution, then yellow precipitate appeared. d) Wagner's test: Few drops of Wagner's reagent were added to 2-3 mLof test solution, and then the reddish-brown precipitate appeared.

7. Tests for Tannins and Phenolic Compound: Few drops of % Ferric chloride solution were mixed with 2-3 mL of test solution, deep blue-black colour appeared after the reaction. b) addition of a few drops of Lead acetate solutionin the test solution gave a white precipitate. c) Addition of gelatin solution in test solutionin the test solution gave a white precipitate. d) Few drops pf bromine water: Discoloration of bromine water.

8. Test for vitamins: a) Test for vitamin A: Dissolved a quantity equivalent to 10-15 units in 1mL chloroform then 5 mL of antimony trichloride solution was mixed, and a transient blue colour was produced immediately. b) Test for vitamin C (Ascorbic acid): Diluted 1 mL of 2% w/v solution with 5mL of water and added 1 drop of freshly prepared 5% w/v solution of sodium nitroprusside and 2 mL dilute NaOH solution. After that 0.6 mL of hydrochloric acid was added dropwise and stirred, and the yellow colour turns blue. c) Test for vitamin D: Dissolved a quantity of test solution equivalent to about 100 units of vitamin D in chloroform then added 10 mL of antimony trichloride solution, a pinkish-red colour appeared at once.

9. Tests for glycosides: Part A: To 2-3 mL of test solution, a few drops of dilute H_2SO_4were added and heated in a water bath for 1-2 mins. Neutralised with 10% NaOH, checked with litmus paper and to the resulting solution Fehling's A& B was added. Increased red precipitate showed the presence of glycosides. Part B: To 2-3 mL of test solution water was mixed and heated. After that NaOH was added to neutralize and mixedwith an equal quantity of water. To the resulting solution, Fehling's A & B solutions were added. Increased red precipitate showed an absence of glycosides. Compare Part A and B. Tests for Cardiac Glycosides a) Baljet's test: A test solution observed for yellow to orange colour with sodium picrate. b) Legal's test (For cardenoloids): To test solution 1 mL of pyridine and 1 mL of sodium nitroprusside were added, and reaction developed a pink to red colour. c) Test for deoxysugars (Kellar Killani test): To 2 mL test solution 3 mL of glacial acetic acid was mixed, then added one drop of 5% $FeCl_3$ and concentrated H_2SO_4along the side of the test tube reaction mixture gave reddish-browncolour at the junction of the two liquid and upper layers turned into bluish-green colour. d) Libermann's test (For bufadenolids): Boiled the mixture of 3 mL of the test solution and 3 mLof acetic anhydride in test tube and cooled. After that, a few drops of concentrated H_2SO_4 were added to develop blue colour.

Tests for Saponin glycosides: a) Foam test: The test solution was shaken vigorously with water. Persistent foam was observed.

10. Test for Triterpenoids: a) Salkowaski test: A few drops of con. Sulphuric acid were added to the test solution, shaken and allowed to stand, lower layer turns yellow. b) Liebermannn Burchardt test: The test solution treated with acetic anhydride, then mixed well and con. Sulphuric acid was added from the sides of the test tube. Deep red colour developed.

Results and discussion

Physico-chemical characterization of Ajwain extracts

Various physiochemical tests were carried out to check out the physical appearance, odour, colour, taste (Organoleptic test) and extractive value of seed extracts in various solvents.Under the organoleptic testin ajwain the nature was coarse, brown colour gives characteristic odour and taste is bitter.

Hexane solvent showed maximum extractive value for ajwain (16.28) followed by alcohol (16.04) and methanol (14.75) solvent. Hence, for further clinical trials, hexane and methanol extracts were selected. Total ash content of the ajwain seed was 10% of which acid insoluble ash was 2% and water soluble ash 1.4% (table 1).

Table 1. Physico-chemical characterization of *Ajwain* extracts

S.N.	Parameters	Observations Ajwain
I	**Physical Tests**	
	Nature	Coarse Powder
	Color	Brown
	Odour	Characteristic
	Taste	Bitter
II	**Extractive Values**	
	Aqueous	12.47
	Alcohol	16.04
	Hydro -Alcoholic (70:30)	14.26
	Benzene	14.38
	Petroleum Ether	08.53
	Hexane	16.28
	Methanol	14.75
III	**Loss on Drying**	8.2
IV	**Ash Values**	
	Total Ash	10
	Acid Insoluble Ash	2
	Water Soluble Ash	1.4

Preliminary phytochemical investigation of methanol and benzene extracts of Ajwain seeds.

When advance techniques for phytochemical analysis are unavailable or unaffordable, conventional phytochemical tests remain a good choice for preliminary phytochemical screenings since they are easy, inexpensive, and require few resources. Qualitative chemical tests were conducted to identify various phytoconstituents in all samples. Various tests were performed to identify carbohydrates, proteins, steroids, amino acids, flavonoids, alkaloids, tennis, vitamin, glycosides and triterpenoids in ajwain. In ajwain carbohydrate, protein, amino acid, steroids, and flavonoids were detected in both the solvent and all the tests were positive. Alkaloids, Triterpenoids and vitamin tests were absent in both the solvent of ajwain. Tenins and glycosides were present in some test of methanol extract and also hexane extract of ajwain.

Since these tests were qualitative, they showed the absence or presence of particular compounds. However, quantitative analysis of tested compounds is necessary to detect minute amounts present in the extract that are undetectable by qualitative analysis (table 2).

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Table 2. Preliminary phytochemical investigation of methanol and benzene extracts of selected plants

S.N.	NAME OF THE TEST		OBSERVATION Ajwain	
			Methanol	Hexane
I	**Test for Carbohydrates**			
	a.	Molisch's test	+	+
	b.	Fehling's test	+	+
	c.	Benedict's test	+	+
	d.	Barfoed's test	+	+
	e.	Cobalt-Chloride test	-	-
	f.	Test for non-reducing sugar	+	+
II	**Test for Proteins**			
	a.	Biuret test	+	+
	b.	Million's test	+	+
	c.	Xanthoproteintets	+	+
	d.	Test for protein containing sulphur	-	-
	e.	Precipitation test	+	+
III	**Test for steroids**			
	a.	Salkowski reaction	+	+
	b.	Liberman- Burchard	+	+
	c.	Liberman's reaction	-	-
IV	**Test for amino acids**			
	a.	Ninhydrin test	+	+
	b.	Test for tyrosine	+	+
	c.	Test for tryptophan	+	+
V	**Test for flavonoids**			
	a.	Shinoda test	+	+
	b.	Lead acetate test	+	+
	c.	Alkaline solution test	+	+
	d.	Ferric chloride test	+	+
VI	**Test for alkaloids**			
	a.	Dragendroff test	-	-
	b.	Mayer's test	-	-
	c.	Hager's test	-	-
	d.	Wagner test	-	-
VII	**Test for Tannins**			
	a.	Lead acetate test	+	+
	b.	5% FeCl3 test	+	+
	c.	Gelatin solution	-	-
	d.	Bromine water	-	-
VIII	**Test for Vitamins**			
	a.	Test for Vitamin A	-	-
	b.	Test for Vitamin C	-	-
	c.	Test for Vitamin D	-	-
IX	**Test for Glycosides**			
	a.	Part a test	+	+
	b.	Part B test	-	+
	c.	Beljet's test	+	+
	d.	Legal's test	+	-
	e.	Keller killani test	-	-
	f.	Libermann's test	-	-
	g.	Foam test	-	-
X	**Test for Triterpenoids**			
	a.	Salkowaski test	-	-
	b.	Liebermann Burchardt test	-	-

Where + denotes present and – denotes absence of phytochemical

varying sensitivity to antibiotics. *Lett. Appl. Microbiol.* 47: 167-173.

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