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Interactions of secondary metabolites from Adenocalymma alliaceum in inhibiting microbial growth

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ABSTRACT

Methanol, ethanol, chloroform, acqueous extracts of Adenocalymma alliaceum leaves were active against selected bacteria. Methanol extract exhibited maximum inhibition followed by ethanol. The 16 fractions obtained after chromatographic separation of methanol extract, are mixed according to taguchi experimental design and checked for antibacterial activity. The values of zones of inhibition are submitted to the software. The combinations of the fractions showing maximum inhibition are Fraction K x Fraction I, Fraction K x Fraction G, Fraction G x Fraction J, Fraction F x Fraction I. The least in: Fraction B x Fraction D, Fraction D x Fraction E, Fraction D x Fraction J.

Keywords:Adenocalymma alliaceum, antibacterial activity, column chromatography,taguchi methodology

Introduction

Plants are used as source for curing ailments since time immemorial. Indian scriptures like the Rigveda, Atharvanaveda, Ayurveda and Charaka samhitha contain a comprehensive record of medicinal plants and their uses. Modern therapy now tends to use the active ingredients of plants rather than the whole plants. The phytochemicals may be synthesized, compounded or otherwise transformed to make pharmaceuticals. Examples of such derivatives include Digitoxin from digitalis; capsaicine from chilli; and aspirin which is chemically related to the salicylic acid found in white willow. Plants indigenous to India like Azadirachta, Curcuma, Ocimum have attracted their attention of international community for their sought after medicinal applications.

According to WHO, more than 80% of the world's population relies on traditional medicines for their primary health care needs. The medicinal value of plants lies in some chemical substances that produce a definite physiologic action on the human body. The most important of these bioactive compounds of plants are alkaloids, flavonoids, tannins and phenolic compounds. The phytochemical research based on ethno-pharmacological information is generally considered an effective approach in the discovery of new anti-infective agents from higher plants (Duraipandiyan, Ayyanar and Ignacimuthu, 2006). The use of medicinal plants as a source for relief from illness can be traced back over five millennia to written documents of the early civilization in China and India.

Phytochemistry is a distinct discipline somewhere in between organic chemistry, plant biochemistry and closely related to natural products. Not only their chemical compounds such as carbohydrates, protein, and lipids that are used as food by man, but also a multitude of compounds like glycosides, alkaloids, flavonoids, etc. are used as medicines by him in various ways and means. The qualitative and quantitative estimation of the phytochemical constituents of a medicinal plant is considered to be an important step in plant research (Kokate, 1994).

Phytochemical progress has been aided enormously by the development of rapid and accurate methods of screening plants for particular chemicals (Banso and Adeyemo, 2007). Medicinal plants contain physiologically active principles that over the years have been exploited in traditional medicine for the treatment of various ailments (Adebajo, 1983). The drugs contained in medicinal plants are known as active principles. Cowmann, (1999) and Banso and Olutimayin (2001) reported that plants contain a wide variety of active principles. There is a reasonable likelihood that medicinal plants with a long history of human use will ultimately yield novel drug prototypes (Eshrat and Hussain, 2002).

Antiaflatoxigenic potency of *Adenocalymma alliaceum* on fungi causing biodeterioration of food commodities and raw herbal drugs was carried out by Ravindra shukla, 2008.

In spite of its uses against cold, fever, pain and inflammation of arthritis and rheumatism, it still has little application in phytotherapy when compared to garlic (Allium sativum). (Chaves & Reinhard, 2006). Antimicrobial activity of some important medicinal plant against plant and human pathogens was reviewed by JL Rios and MC Recio, 2005.

Application of taguchi method for the optimization of interactions in the plant solvent extractions was studied by Ugur , 2009. In taguchi method the interactions are studied by using the analysis of variance (ANOVA).

Essential oils extracted from the leaves of Adenocalymma alliaecium, has been found to inhibit in-vitro growth of E. coli, B. anthracisand P. vulgaris showing its antibacterial activity. This was reviewed by Unander et al., in 1990. Plants have evolved multiple defense mechanisms against microbial pathogens and various types of environmental stress. Besides anti-microbial secondary metabolites, some of which are preformed and some of which are induced by infection has been reviewed by Mazid , Khan and Mohammad in 2011.

Materials And Methods

Preparation of leaf extract for antimicrobial activity

The leaves of Adenocalymma alliaceum and air dried. It was then ground to powder using an electric mill. The powdered material (10 g) was subjected to Soxhlet extraction for 18h using water, methanol, ethanol, chloroform and hexane separately. The extracts were concentrated and further used for anti microbial activities.

Assay for antibacterial activity
The bacteria were grown on Nutrient agar at 37°C. They were then maintained on nutrient agar slants at 4°C and stored at -20°C. Inoculum of test bacterial species was prepared by growing pure isolate in nutrient broth at 37°C for overnight. The broth cultures were sub cultured in fresh nutrient broth, for 3h to obtain log phase culture. The agar plates were prepared by pour plate method using 20 ml nutrient medium.

The molten sterile nutrient agar medium was cooled to 45°C and mixed thoroughly with 1ml of growth culture of concerned test organism (1 x 108 cells) and then poured into the sterile Petri plates (100x17mm) and allowed to solidify. Wells of 6mm size were made with sterile cork borer and 5, 25 and 50 µl of extracts were added. The agar plates were incubated at 37°C for 24h. The diameters of zones of inhibition were measured in mm using Himedia Zone reader.

Separation of plant extract by Adsorption chromatography
The methanol extract was separated by Adsorption chromatography using alumina as the support medium and benzene acetone mixture as the elution solvent. Different fractions were collected and wavelength scan (200-700nm) was carried out using UV-Visible spectrophotometer. The fractions exhibiting same peak patterns were pooled and checked for antimicrobial activity by the spread plate technique.

Interaction studies
Studies on the interaction of the individual sample components exhibiting antimicrobial activity is carried out with the help of QUALITEK 4 software which works on the principle of Taguchi method.

Results
The effect of Adenocalymma alliaceum leaf extracts on bacterial growth is shown in table 1. The methanol, ethanol, chloroform, extracts were active against Escherechia coli, Klebsiella pnuemoniae, Bacillus subtilis, Staphylococcus aureus, Streptococcus marceius and Proteus vulgaris where as, the aqueous extract was ineffective against Bacillus subtilis.

Methanol extract exhibited maximum zone of inhibition against all the test organisms among all the extracts used. A significant inhibition of 12mm was exhibited against Klebsiella pnuemoniae and Proteus vulgaris.

Ethanol extract exhibited maximum zone of inhibition after the methanol extract. A significant inhibition of 8mm was exhibited against all the organisms except Staphylococcus aureus and Streptococcus marceius.

As stated, the aqueous extract was ineffective against Bacillus subtilis but, it showed the maximum activity of 9mm against Streptococcus marceius.

The chloroform extract showed the least activity amongst all the extracts. A maximum inhibitory activity of 5mm is observed against Proteus vulgaris.

Table1: Anti-microbial activity of different extracts of Adenocalymma alliaceum leaf

	METHANOL EXTRACT zone of inhibition in cm	ETHANOL EXTRACT zone of inhibition in cm	AQUEOUS EXTRACT zone of inhibition in cm	CHLOROFORM EXTRACT zone of inhibition in cm
E.coli	0.5,0.5, 0.6,1.0	0.2,0.4, 0.6,0.8	0.2,0.4, 0.5,0.6	-,0.1,0. 3,0.4
K.pnuemonia	0.3,0.6, 0.8,1.0	0.2,0.4, 0.6,0.8	0.2,0.4, 0.6,0.8	-,-,0.2, 0.4
B.subtilis	10.4,0.8, 1.0,1.2	0.2,0.4, 0.6,0.8	-,-,-,-	-,0.1,0 .2,0.4
S.aureus	0.2,0.4, 0.8,1.0	0.2,0.3, 0.4,0.6	0.1,0.2, 0.3,0.4	-,0.2,0. 3,0.4
S.marceius	0.4,0.5, 0.6,0.8	0.2,0.3, 0.4,0.6	0.3,0.4, 0.6,0.9	-,-,0.1, 0.4
P.vulgaris	0.4,0.6, 0.8,1.2	0.2,0.4, 0.6,0.8	0.3,0.6, 0.6,0.6	-,0.2,0. 3,0.5

Results obtained in the above study reveals that the methanol extract possessed antibacterial activity against E. coli, Klebsiella pnuemoniae, Bacillus subtilis tested with the spread plate method. Among different microbial species the methanol extract of plant showed significant activity against Bacillus subtilis.

As the highest amount of activity is shown against Bacillus subtilis, methanol extract is separated through column chromatography. The fractions thus obtained from the column chromatography are analysed through spectrophotometric method and the fractions which shows the similar peaks are pooled and are concentrated on water bath. These concentrated samples are checked for the anti-microbial activity (table 2).

Table 2: Inhibitory activity of column chromatographic samples against Bacillus subtilis

S.NO	Zone of inhibition
Fraction 1	10mm
Fraction 2	13mm
Fraction 3	6mm
Fraction 4	10mm
Fraction 5	20mm
Fraction 6	18mm
Fraction 7	8mm
Fraction 9	13mm
Fraction 10	6mm
Fraction 11	14mm
Fraction 12	15mm

As there is no significant inhibition in the individual fractions, they are mixed according to taguchi method and checked for the contribution of factors and the interactions.

After mixing all the 12 fractions according to the taguchi experimental design (table-3) 16 fractions are obtained which are checked once again for the antibacterial activity.

Table 3: The column fractions are mixed according to the taguchi methodology.

	A	B	C	D	E	F	G	H	I	J	K	L	M	N	O	P
1	1	1	-	1	-	-	1	1	1	1	1	1	1	1	1	-
2	1	1	-	1	-	-	1	2	2	2	2	2	2	2	2	-
3	1	1	-	2	-	-	2	1	1	1	1	2	2	2	2	-
4	1	1	-	2	-	-	2	2	2	2	2	1	1	1	1	-
5	1	2	-	1	-	-	2	1	1	2	2	1	1	2	2	-
6	1	2	-	1	-	--	2	2	2	1	1	2	2	1	1	-
7	1	2	-	2	-	-	1	1	1	2	2	2	2	1	1	-
8	1	2	-	2	-	-	1	2	2	1	1	1	1	2	2	-
9	2	1	-	1	-	-	2	1	2	2	2	1	2	1	2	-
10	2	1	-	1	-	-	2	2	1	1	1	2	1	2	1	-
11	2	1	-	2	-	-	1	1	2	2	2	2	1	2	1	-

12	2	1	-	2	-	-	1	2	1	1	1	1	2	1	2	-
13	2	2	-	1	--	-	1	1	2	1	1	1	2	2	1	-
14	2	2	-	1	-	-	1	2	1	2	2	2	1	1	2	-
15	2	2	-	2	-	-	2	1	2	1	1	2	1	1	2	-
16	2	2	-	2	-	-	2	2	1	2	2	1	2	2	1	-

The16 fractions obtained from the taguchi method are checked once again for the antimicrobial activity. The results obtained from the antimicrobial activity are submitted to the qualitek4 software, it shows the results in terms of

1. Significant factors
2. F ratio, pure sum %
3. Interactions severity index
4. Total contribution from all factors
5. Current guard average of performance
6. Experimental results at optimum conditions

Anti bacterial activity of fractions obtained through taguchi method(table 4).

Table 4: Zone of inhibition of interacting samples against Bacillus subtilis:

S.NO	ZONE OF INHIBITON
Fraction 1	28mm
Fraction 2	23mm
Fraction 3	20mm
Fraction 4	15mm
Fraction 5	18mm
Fraction 6	25mm
Fraction 7	19mm
Fraction 8	11mm
Fraction 9	18mm
Fraction 10	19mm
Fraction 11	15mm
Fraction 12	18mm
Fraction 13	17mm
Fraction 14	12mm
Fraction 15	16mm
Fraction 16	13mm

These values of zone of inhibition are submitted to the software.

The 2 types of fraction volumes are taken 20 and 40µl of samples because these are known to be the significant volumes below which there is no significant activity.

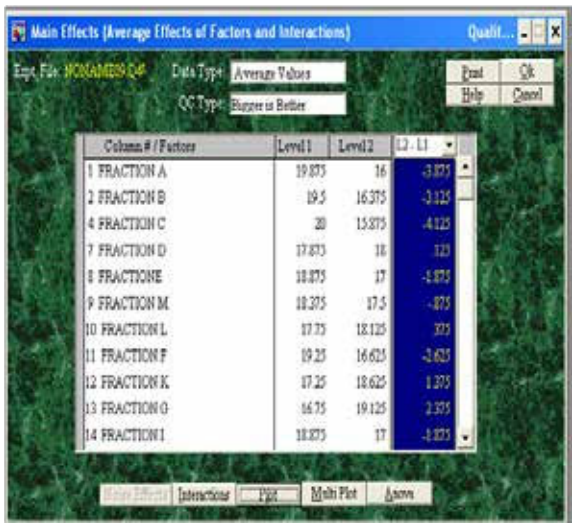


Fig 1: Average effects of factors and figures

Fig 1 is showing the significant activity of both level1 and level 2 and the difference between those levels. Fraction A and Fraction G in level1 and 2 respectively are exhibiting significant activities.

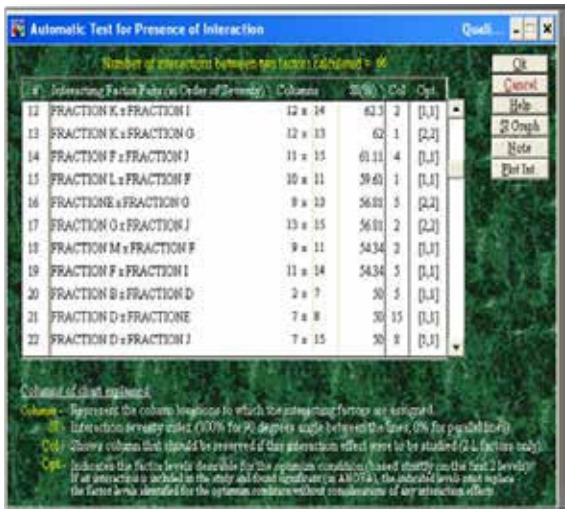
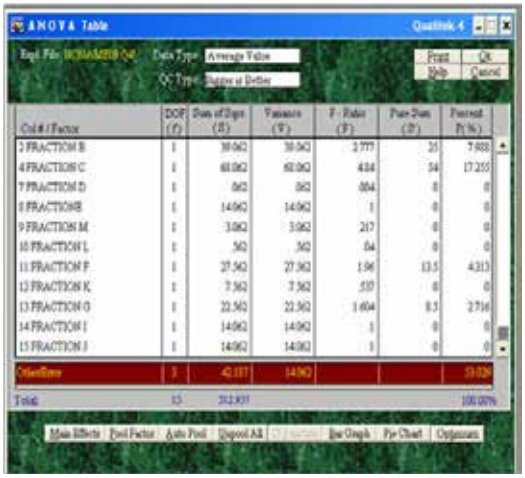


Fig 2: Automatic test for presence of interactions

Even if it is not showing any significant inhibition in individual fractions the combinations of the fractions showing the maximum inhibition(Fig 2). Maximum activity is seen in: Fraction K x Fraction I, Fraction K x Fraction G, Fraction G x Fraction J, Fraction F x Fraction I. The least is seen in: Fraction B x Fraction D, Fraction D x Fraction E, Fraction D x Fraction J.

Fig 3: ANOVA for various fractions



The variance; percentage of significance; psum are shown in fig 3.

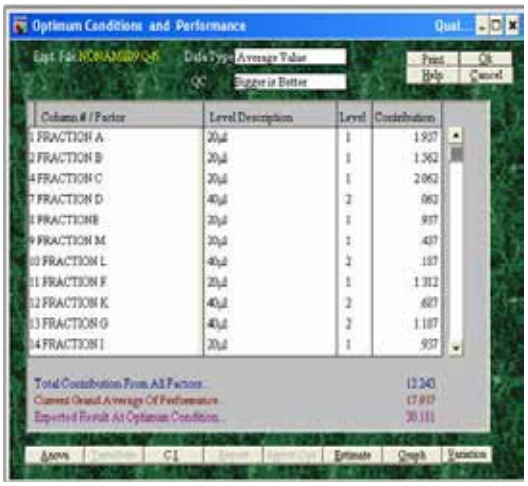


Fig 4: Optimum conditions and performance

Fig 4 shows that, what level is best contributing out of the 2 levels we have for the experiment. In some fractions level1 is showing the significant inhibition but in some fractions level 2 is showing.

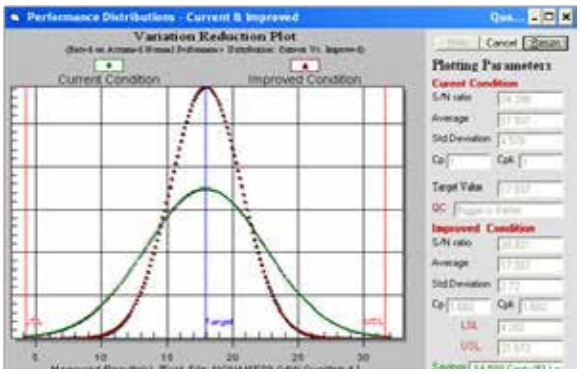


Fig 5: Graph showing performance distribution

The graph is showing the difference between conditions where the single fraction is used and where the interactions of the different fractions are used.

Discussion

The methanol, ethanol, chloroform and aqueous extracts of the leaves of Adenocalymma alliaceum was obtained by soxhlet extraction. Methanol extract exhibited maximum inhibition against all the tested bacteria. In order to identify the active principle methanol extract was separated into individual fractions on column chromatography and tested for antibacterial activity. As no significant activity was exhibited by individual fractions interaction studies were carried out using software called as QUALITEK 4. The samples pooled according to automatic experimental design were again checked for antibacterial activity. Due to combinatorial action of the components significant antibacterial activity was established.

Among all the pooled fractions significant activity is seen in the fractions A and G. The maximum severity index of 86% is seen in the combinations D and L and the least of 61% is seen in the combinations G and J. The maximum variance of 39.06 is seen in the fraction B which is the major contributing factor among all the other fractions. According to the variation reduction plot there is a slight difference between the ideal result and the experimental result.

From the studies carried out the interaction of the various principles present in the extract play a major role in inhibiting the growth of microorganisms rather than individual action. The interactions of these compounds need to be further explored.

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