ISSN NO. 2320-5407

Journal homepage: http://www.journalijar.com

INTERNATIONAL JOURNAL OF ADVANCED RESEARCH

RESEARCH ARTICLE

ANTIBACTERIAL AND PHTYOCHEMICAL ANALYSIS ON BRASSICA OLERACEAE VAR ITALICA.

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Manuscript Info

Manuscript History:

Received: 14 January 2016
Final Accepted: 18 February 2016
Published Online: March 2016

Key words:
Brassica oleraceae, Brassicaceae, Antibacterial, Phytochemical analysis, HPLC and FT-IR analysis.

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Abstract

Objective: - To investigate the antibacterial activity and phytochemical analysis of Brassica oleraceae var. italica

Methods: - The antibacterial activity was evaluated using agar well diffusion and microdilution methods against the bacterial (E.coli, Proteus, S.aureus, Klebsiella and Pseudomonas) iolates. The extraction of the vegetable was carried using the solvent namely acetone. Phytochemical, FT-IR and HPLC analysis was carried out in the acetone extract.

Results:-Acetone extract showed maximum activity against E.coli, Proteus, S.aureus, Klebsiella and Pseudomonas. Phytochemical analysis of Brassica oleraceae extracts showed the presence of secondary metabolites like alkaloids, flavonoids, glycosides, phenolic compounds, saponins, tannins, terpenoids, carbohydrates and anthraquinone. The FT-IR and HPLC revealed different characteristic peak values with various functional compounds in the extracts.

Conclusion: - From this study, it can be concluded that Brassica oleraceae var. italica exhibits antibacterial activity against certain microorganisms.

Introduction:-

History and Botany:-

Broccoli is the annual green heading form of Brassica oleracea L. var. italica Plenck grown in Australia, New Zealand, Japan, the United States of America, Canada, Germany, and The Netherlands since the late 1980s. The same green heading form of broccoli is known as Calabrese (a derivation from Calabria in southern Italy) in the United Kingdom and Italy (Gray 1982, 1989, Titley 1985). Before the advent of popular heading forms, the term broccoli was used to describe the green sprouting form (see Fig. 2.1) (Nieuwhof 1969, Seelig 1971, Titley 1985). The green sprouting form produced most of its yield from multiple harvests of lateral heads or sprouts whereas the heading form produced a large, single, terminal inflorescence.

Broccoli is thought to have originated in the eastern Mediterranean. The crop was introduced into Italy where crop diversification took place and open pollinated, sprouting and coloured heading forms were developed. Broccoli was introduced to Western Europe in the 18th century and North America in the 20th century (Gray 1982). Development of single heading hybrids by plant breeders in the United States, Japan, The Netherlands and United Kingdom during the 1970s revolutionized broccoli production in the 1980s and 1990s (Shinohara 1984, Titley 1985).

Broccoli belongs to the family Brassicaceae, which includes other vegetable crops such as cauliflower (B. oleracea L. var. botrytis Alef), cabbage (B. oleracea L var. capitata Alef.), Brussels sprouts, kohl-rabi and kale. The distinction between broccoli and cauliflower can be made by their comparative morphology at the harvestable stage (Fig. 2.1). At harvest, the surface of the broccoli head is made up of a mass of fully differentiated floral buds whereas the cauliflower head (curd) is a dome of tissue made up of a mass of floral primordial meristems (Sadik 1962, Malatesta and Davey 1996). Marketable broccoli is ontogenetically more mature than marketable cauliflower. Most broccolis are green in colour due to chlorophyll within the sepals of the floral buds. This contrasts with the white or cream colour curd in cauliflower which lacks chlorophyll (Gray 1982, 1989, Shinohara 1984).

Broccoli - Brassica oleracea L. var italica Plenk - belongs to family Brassicaceae. The word **"Brassica"** means to cut off the head. Broccoli is an Italian word from the Latin **"brachium** "meaning an arm or branch. Broccoli is fast becoming an important fresh market and processing vegetable crop in many parts of the world (Morelock, Peerson and Motes, 1982; Magnifico, Lattanzio and Sarli, 1979).

The term sprouting as used in sprouting broccoli refers to the branching habit of this type, the young edible inflorescences often being referred to as sprouts. The sprouting broccolis are thought to have originated from the eastern Mediterranean then introduced into Italy. A remarkable diversity of broccoli-like vegetables has been developed in Italy. According to (FAO statistics, 2012), China is the top world producer of broccoli (9,596,000 tons). The flowers of broccoli are borne on a faceted floral shoot so that the inflorescence terminates the axis of the plant. The inflorescence, which has been described as a corymb, a corymbose panicle or a modified racemose panicle, consists of functional floral buds, perfect flowers, stem and bracts. At the time of harvesting, the inflorescence is a growing, faceted axis bearing a large number of
immature, stalked flowers, floral buds and varied bracts which are smaller and simpler in form than the vegetative leaves. The bracts are absent from the terminal portion of the inflorescence.

Vegetables make up a major portion of the diet of many parts of the world and play a significant role in human nutrition, especially as sources of phyto- nutriceuticals: vitamins (C, A, B1, B6, B9, E), minerals, dietary fiber and phytochemicals. Some phytochemicals of vegetables are strong antioxidants and are thought to reduce the risk of chronic disease by protecting against free-radical damage, by modifying metabolic activation and detoxification of carcinogens, or even influencing processes that alter the course of tumor cells.

Vegetables in the daily diet have been strongly associated with overall good health, improvement of gastrointestinal health and vision, reduced risk for some forms of cancer, heart disease, stroke, diabetes, anemia, gastric ulcer, rheumatoid arthritis, and other chronic diseases. A high vegetable diet has been associated with lower risk of cardiovascular disease in humans. Low vegetable intake, in unbalanced diets, has been estimated to cause about 31% of ischemic heart disease and 11% of stroke worldwide. According to the 2007 World Health Report unbalanced diets with low vegetable intake and low consumption of complex carbohydrates and dietary fiber are estimated to cause some 2.7 million deaths each year, and were among the top 10 risk factors contributing to mortality. The exact mechanisms by which vegetable consumption reduces human diseases have not yet been fully understood, however the general consensus among physicians and nutritionists is that phytonutriceuticals in vegetables are responsible for mitigating some of these diseases.

Vegetables have been analyzed as potent medicine and man is able to obtain from them a wondrous assortment of industrial chemicals. In recent years population continues to explode and microbial disaster may occur. So vegetables with possible antimicrobial activity should be tested against an appropriate microbial model to confirm its activity and to ascertain the parameter associated with it.

Infectious diseases continue to be a serious burden around the world, in developing and industrialized countries alike. The history of prevention and treatment of disease or the science of healing started from ancient period, when it was considered more as an art than a science. A remarkable scientific breakthrough was developed in the 19th & 20th centuries. Now-a-day's multidrug resistance from indiscriminate usage of the antibiotics has led to pursuance of natural drugs. Herbal medicines are becoming popular in modern world as people resort to natural therapies. Natural products isolated from higher plants have been providing novel clinically active drugs which created the scientist to identify the potent and effective antimicrobial agents of plant origin to replace the antibiotics. It is evident that plants produce a diverse range of bioactive molecules which can inhibit the growth of microbes.

Phenological development and ontogeny:-

Phenology is the study of biotic events that occur once in a growing season of a crop (Alm et al. 1991). It describes, and measures developmental and physiological processes controlling growth and development, and the environmental influences. The following seven phenological stages of broccoli were described for crop protection purposes (i.e. control of weed, disease and insect pests) (Theunissen and Sins 1984):

1. Seed stage - Spherical and brownish-black seed.
2. Seedling stage - The seed has germinated and the laminas of the two cotyledons unfold at the top of the hypocotyl. The leaf-sheaths are still united.
3. First leaf stage - The first true leaf develops between the fully extended cotyledons.
4. Transplanting stage - The two first true leaves originate at the same height as the cotyledons. More leaves are formed and the plant grows.
5. Vegetative stage - Axillary buds develop on the leaf sheaths. Older leaves show an axis of about 45o with soil level. Younger leaves stand upright.
6. Harvesting stage - The inflorescence develops from the terminal apex and grows until it has reached its maximum size as a marketable product.
7. Flowering stage - Flowering.
8. Seed production stage - Seed development

Stages 6 and 7 are used mainly for plant breeding and seed production purposes, and are not directly relevant for fresh market broccoli production. Stages 3 and 5 are 'human' imposed stages that have limited biological meaning but are relevant for agronomic purposes. The floral initiation (FI) stage, essential for prediction of harvest dates (Wurr et al. 1991a), was not identified in the stages mentioned above.

Materials and methods:-

Vegetable Collection:-

Brassica oleraceae was collected from the local markets of Trichy, TamilNadu, India. The vegetable was washed thoroughly under running tap water to remove dirt and then shade dried at room temperature for a week. They were ground into fine particles after drying and kept in closed container before being stored at room temperature until further used. The date, place and information of vegetable collection were recorded.

Extraction and sample preparation:-

Broccoli vegetables were purchased from the local market and the florets were removed from the head, dried, pulverized and extracted with solvents of increasing polarity such as acetone at room temperature for 48 hours. The extracts were filtered using Whatman No.1 filter paper and concentrated to dryness under reduced pressure in a rotary evaporator and stored in sterile vials at 4ºC until used.

Test organisms:-

Bacillus species, Escherichia coli, Staphylococcus aureus, Proteus species and Pseudomonas species.

Antimicrobial assay:-

Well diffusion method:-

The agar well diffusion method was employed for the determination of antimicrobial activity of the extracts. To brief, wells were made in Muller Hinton agar plates (Himedia, Mumbai, India) using cork borer (5mm diameter) and the inoculums containing 50 µl of bacteria and fungi were swabbed on the above plates with a sterile swabs separately. 20 µl of the Brassica oleracea extracts, control (DMSO) and standard antibiotics (4mg of Chloramphenical and nystatin) (Himedia, Mumbai, India) was filled in wells with the help of micropipette separately. The plates were then incubated at 37º C for 24 hours for bacteria and at room temperature (25 -30º C) for five days for fungal strains. The samples were tested in duplicates and the diameter for the zone of inhibition was measured as millimeter (mm).

Disc diffusion method:-

The disc diffusion method was determined by micro dilution method using the serially diluted Brassica oleraceae extracts. The acetone extract was diluted to different concentrations in sterile Muller Hinton broth. The microorganism suspension of 50µl was added to the broth dilutions and was incubated at 37ºC for 24 hours. The

MIC values were taken as the lowest concentration of the extract in the well of the microtitre plate that showed no turbidity after incubation. The turbidity of the wells in the microtitre plate was interpreted as visible growth of microorganisms.

Phytochemical analysis:-

Phytochemical analysis of the solvent extracts of Broccoli was performed by the following standard procedures (Trease and Evans, 2002; Harborne, 1998 and Sofowara, 1993). In brief, 0.5 ml of extract was added with a drop or two of Mayer's reagent by the side of the test tube and the formation of white or creamy precipitate indicates the presence of alkaloids.

- Adding 1ml of extract with ammonia and conc. Sulphuric acid and disappearance of yellow colour on standing indicates flavonoids.
- Formation of brown ring at the interface by the addition of 2ml of glacial acetic acid followed by few drops of ferric chloride solution and 1ml of conc.Sulphuric acid to the extracts revealed the presence of glycosides.
- Adding few drops of neutral ferric chloride to the extract and the development of dark green colour indicates the presence of the phenolic compounds
- Existence of froth formation during warming and vigorous shaking indicates saponins.
- Appearance of brownish green or blue black coloration after adding 0.1% ferric chloride to the cooled extract indicates tannins.
- Addition of 2ml of chloroform and 3ml of conc. Sulphuric acid to the extract and the formation of reddish brown layer at the junction of two solutions confirms terpenoides.
- Boil the extract with the little amount of Benedict's solution; if glucose is present the colour change from blue to opaque green, then to yellow and finally to red indicates the presence of carbohydrates.
- Boil the test material with 1ml of dil.sulphuric acid in test tube for 5 minutes, centrifuge and filter while hot, filtrate, cool and shake with an equal volume of dichloromethane which separates the lower dichloromethane layer and shake with half of its volume with dil.ammonia , the red colour is produced in the ammonical layer indicates anthraquinone.

FT-IR Analysis:-

FT-IR analysis (Fourier Transform Infrared) is perhaps the most powerful tool for identifying the types of chemical bonds (functional groups). The wavelength of light absorbed is characteristic of the chemical bond which can be seen in the annotated spectrum. By interpreting the infrared absorption spectrum, the chemical bonds in a molecule can be determined.

For the FT-IR study, dried powder of acetone extract (10mg) of Brassica oleraceae was taken in a mortar and pestle and ground with 2.5mg of dried potassium bromide (KBr). The powder so obtained was filled in a 2mm internal diameter micro-cup and loaded onto FT-IR set at 26ºC ± 1ºC. The samples were scanned using infrared in the range of 3500-500 cm^{-1} using Fourier Transform Infrared Spectrometer (Shimadzu, IR Affinity 1, and Japan). The spectral data obtained were compared with the reference chart to identify the functional groups present in the sample.

HPLC Analysis:-

The High-performance liquid chromatography coupled with diode array detector (HPLC-DAD) analysis of polyphenolic compounds of vegetable extracts was measured according to the existing method in the laboratory, (Jaiswal et al.,2011a).

In brief, the HPLC system consisted of a reversed-phase HPLC column on an Alliance HPLC (e2695 separations modules; waters, Milford, MA, USA) equipped with an auto sampler and controller with dual pump, a 2998 photodiode array detector and the Empower software.

An Atlantis C18 column (250x4.6mm, 5µm particle size) from waters was used for the polyphenolic separation at 25ºC. Mobile phase used was similar to as reported in earlier studies (Jaiswal et al., 2011a).

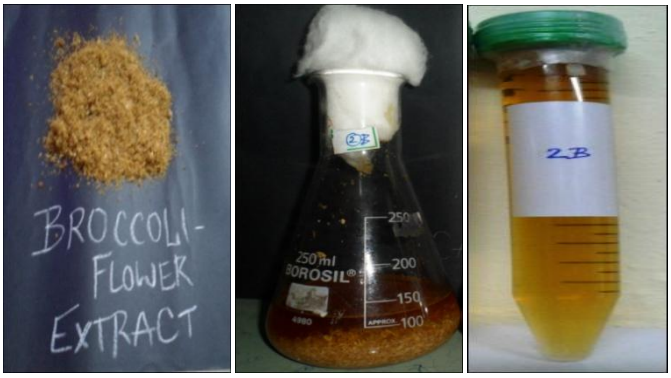
The chromatograms ware monitored at 280nm [hydroxy benzoic acid (HBA)], 320nm [hydroxy cinnamic acid (HCA)], 360nm (flavonea and flavonols) and 520nm (anthocyanins); complete spectral data were recorded in the range of 220-600nm.

The HPLC-DAD recorded UV-vis spectrum of each peak of the chromatogram which allowed explicit attribution of each chromatographic peak to distinct class of PPs as each class exhibits a characteristic UV-vis spectrum. Different groups of PPs were identified by comparing their UV-vis spectra with spectra of reference compounds and reported values. Polyphenolic profiles at 280nm for the studied Brassica vegetables are presented here.

Results:-

Broccoli:-

Phytochemical analysis of the solvent extracts of broccoli was performed by the following standard procedures (Trease and Evans, 2002; Harborne, 1998 and Sofowara, 1993).



Antibacterial assay:-

Well diffusion method

The agar well diffusion method was employed for the determination of antimicrobial activity of the extracts. To brief, wells were made in Muller Hinton agar plates (Himedia, Mumbai, India) using cork borer (5mm diameter) and the inoculums containing 50 µl of bacteria and fungi were swabbed on the above plates with a sterile swabs separately. 20 µl of the Brassica oleracea extracts, control (DMSO) and standard antibiotics (4mg of Chloramphenical and nystatin) (Himedia, Mumbai, India) was filled in wells with the help of micropipette separately. The plates were then incubated at 37º C for 24 hours for bacteria and at room temperature (25 -30º C) for five days for fungal strains. The samples were tested in duplicates and the diameter for the zone of inhibition was measured as millimeter (mm).

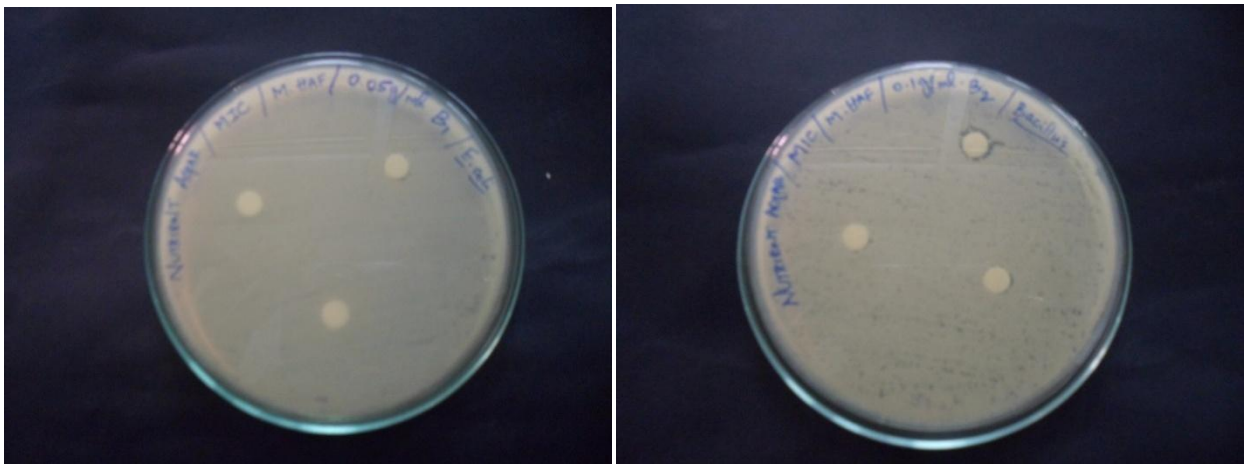


Table.2:- zone diameter of inhibition (mm) in MIC by well diffusion assay (µg/ML) against the gram negative bacteria.

SAMPLE	Zone of inhibition (mm) - Acetone
B1 (E.coli) **0.05(mg/ml)**	22
B2 (Bacillus) **0.1(mg/ml)**	28

Microdilution method:-

The minimum inhibitory concentration (MIC) was determined by micro dilution method using the serially diluted Brassica oleraceae extracts. The acetone extract was diluted to different concentrations in sterile Muller Hinton broth. The microorganism suspension of 50µl was added to the broth dilutions and was incubated at 37ºC for 24 hours. The MIC values were taken as the lowest concentration of the extract in the well of the microtitre plate that showed no turbidity after incubation. The turbidity of the wells in the microtitre plate was interpreted as visible growth of microorganisms.

Antibacterial activity of Brassica oleraceae:-

The results of agar well diffusion method and the microdilution method of Brassica oleraceae using the solvent acetone as depicted in Table 1.

Table1:- Antibacterial activity of the acetonic extracts of Brassica oleraceae uses agar well diffusion method.

Zone of inhibition in diameter (mm)	
Microorganisms	**Acetone**
Escherichia coli	28
Pseudomonas	27
Bacillus	29
Staphylococcus aureus	22
Klebsiella	21

Table 2:- Minimum Inhibitory Concentration (MIC) of Brassica oleraceae against the tested microorganisms

Microorganisms	Acetone extract (mg/ml)			
	40	**20**	**10**	**5**
Escherichia coli	0.52	0.42	0.28	0.20
Pseudomonas	0.28	0.21	0.17	0.19
Bacillus	0.31	0.27	0.21	0.15
Staphylococcus aureus	0.22	0.29	0.20	-
Klebsiella	0.17	0.26	0.21	0.18

The minimum inhibitory concentration (MIC) of the extracts to inhibit the microorganisms was determined by using the microdilution method.

Plate 1: Disc diffusion of Brassica oleraceae extracts by using the bacterial strains.
([1]Bacillus and [2]E.coli)

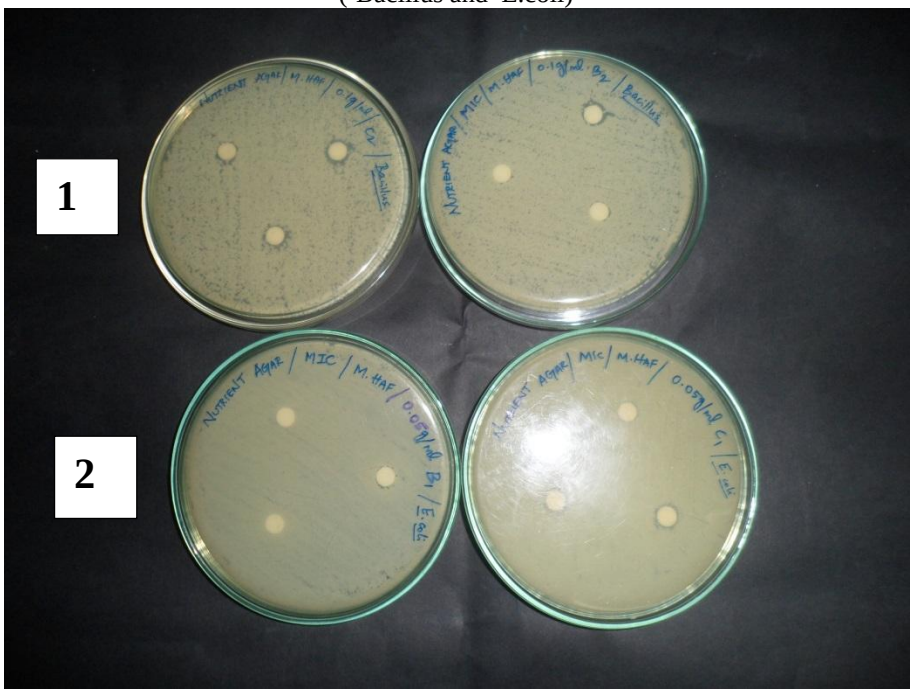


Plate2: Microdilution of Brassica oleraceae extracts by using the bacterial strains.

Table 3:- Qualitative phytochemical analysis of Brassica oleraceae.

Phytochemicals	**Brassica oleraceae extracts**
Extract	**Acetone**
Alkaloids	Positive
Flavonoids	Positive
Glycosides	Negative
Phenolic compounds	Negative
Saponins	Negative
Tannins	Negative
Terpenoids	Positive
Carbohydrates	Negative
Anthraquinone	Negative

Test for alkaloids [Mayer's test]

0.5ml of extract was added with a drop or two of Mayer's reagent by the side of test tube and the **formation of white or creamy precipitates** indicates the presence of Alkaloids

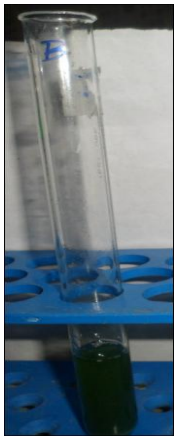
Test for flavonoids [alkaline reagent]

Adding 1ml of extract with ammonia and conc. Sulphuric acid and the **disappearance of yellow colour** on standing indicate flavonoids

Test for glycosides.

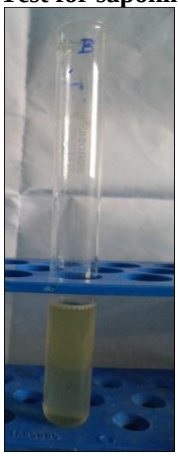
Formation of brown ring at the interface by the addition of 2ml of glacial acetic acid followed by few drops of ferric chloride solution and 1ml of conc.Sulphuric acid to the extracts revealed the presence of glycosides.

Test for phenolic compounds.



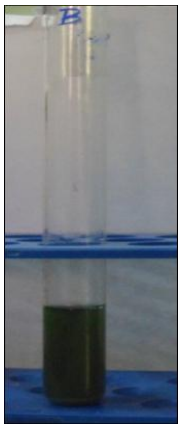
Adding few drops of neutral ferric chloride to the extract and the **development of dark green colour** indicates the presence of the phenolic compounds

Test for saponins [foam test]



Existence of **froth formation** during warming and vigorous shaking indicates saponins.

Test for tannins.

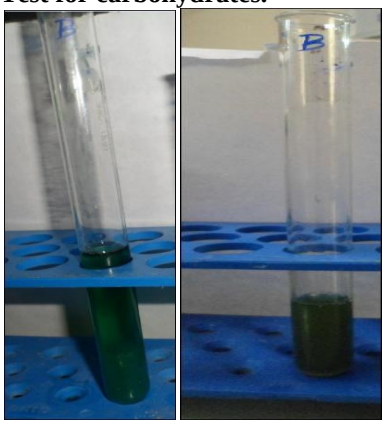


Appearance of **brownish green or blue black coloration** after adding 0.1% ferric chloride to the cooled extract indicates tannins.

Test for terpenoids.

Addition of 2ml of chloroform and 3ml of conc. Sulphuric acid to the extract and the **formation of reddish brown layer** at the junction of two solutions confirms terpenoides.

Test for carbohydrates.



Boil the extract with the little amount of Benedict's solution; if glucose is present the **colour change from blue to opaque green, then to yellow and finally to red.**

Test for anthraquinone [Borntrager's test]

Boil the test material with 1ml of dil.sulphuric acid in test tube for 5 minutes, centrifuge and filter while hot, filtrate, cool and shake with an equal volume of dichloromethane which separates the lower dichloromethane layer and shake with half of its volume with dil.ammonia , **the red colour** is produced in the ammonical layer.

Table 4:- High Performance Liquid Chromatography (HPLC) of Brassica oleraceae var italica extracts.

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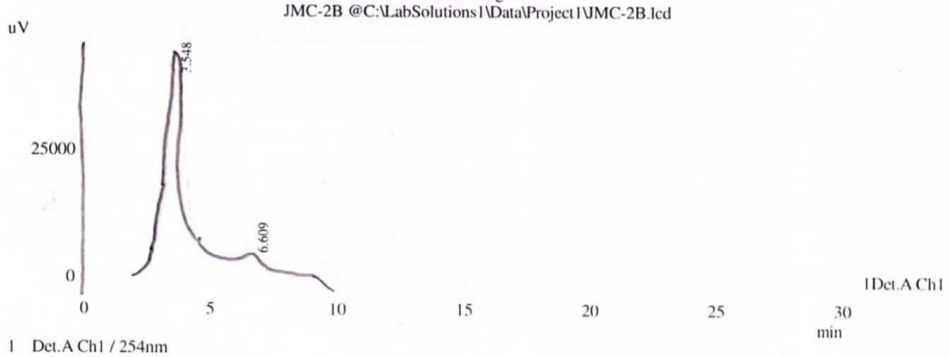
HPLC ANALYSIS REPORT

Sample Information

Acquired by	: Admin
Sample Name	: JMC-2B
Sample ID	:
Vail#	:
Injection Volume	: 20 uL
Data Filename	: JMC-2B.lcd
Method Filename	: JMC-B-Methd.lcm
Batch Filename	:
Report Filename	: Default.lcr
Date Acquired	: 29-01-2016 PM 12:08:28
Data Processed	: 29-01-2016 PM 12:38:31

Chromatogram

JMC-2B @C:\LabSolutions1\Data\Project1\JMC-2B.lcd



1 Det.A Ch1 / 254nm

PeakTable

Detector A Ch1 254nm

Peak#	Ret. Time	Area	Height	Area %	Height %
1	3.548	2629119	44115	88.126	91.826
2	6.609	354256	3927	11.874	8.174
Total		2983374	48042	100.000	100.000

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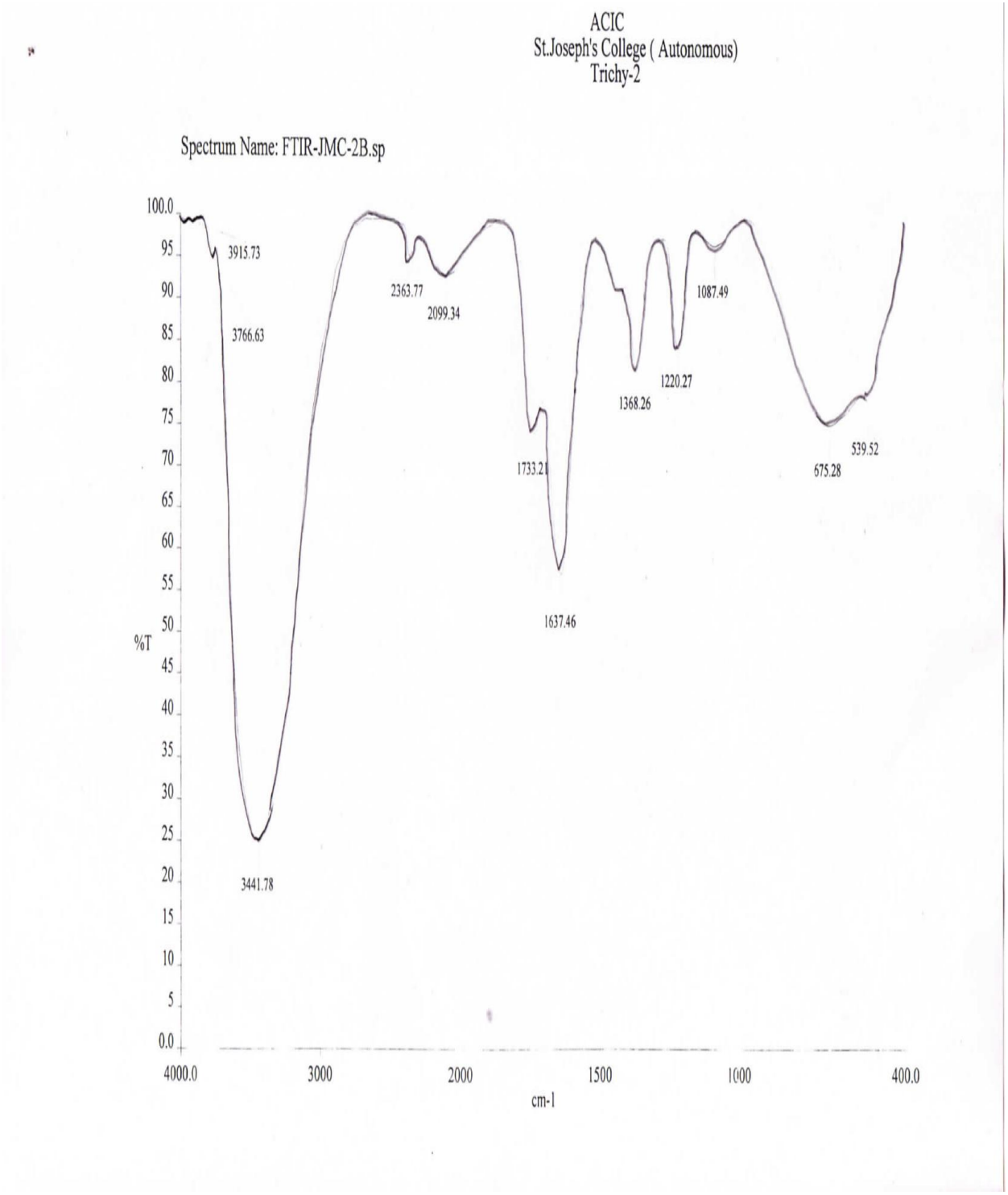
Table 5:- FT-IR analysis of Brassica oleraceae var italica extracts.



Discussion:-

In recent years the use of plants as source of drugs has been increased for the treatment of infectious diseases. Hence, there is a need to move towards the traditional medicine which can serve as novel therapeutics. Numerous studies have highlighted the potential importance of vegetables as a source of medicine which has been inherited as an important component of the health care system in India. Vegetable extracts are given singly or as concoctions for the treatment of microbial diseases. In the present study notable activity was observed against all the tested micro organisms. In an overview of the bioactivity data obtained from the current study, it can be highlighted that the tested extracts have potential to inhibit bacteria and fungi. Pseudomonas aeruginosa exhibited more inhibitory activity which represents the role of phytoconstituents towards the action of permeability on peptidoglycon layer. The maximum antibacterial activity exhibited by the acetone extract may create an acidic environment that caused the disruption of bacterial and fungal cell membrane.. The phytochemical obtained from the vegetables have a potential role in health care industries and also serve as a lead chemicals for new drug development with diverse range of antimicrobial properties. Bioactive substances from this plant can therefore be employed in the formulation of antimicrobial drugs for the treatment of various bacterial and fungal infections. Phytochemical agents act as antimicrobial agent by inhibiting the extracellular enzyme acting on the substrates required for microbial growth or by inhibiting oxidative phosphorylation of microbial metabolism. The phytochemical contents of the leafy vegetables serve as supplements for food and also have the potential to improve the health status of its users as a result of the presence of various compounds vital for good health. Hence, the recent research showed that the complex mixture of phytochemicals in vegetables provides a better protective effect on health than single phytochemicals. Therefore, the complex components of broccoli extract needs to be scrutinized in depth, in order to find out the best mixture of effective components that had role in the currently shown antibacterial and antifungal activity. The presence of characteristic functional groups may be responsible for the medicinal properties of Brassica oleracea var italica which contain high therapeutic content. Determination of respective antimicrobial potential and toxicological evaluation of these extracts with the view to formulate novel chemotherapeutic agents to be used future is worth mentioning.

To conclude, the present bioprospecting study justifies the medicinal uses of the vegetables and also reveals the potentialities to isolate a promising natural compound for the management of the bacterial infectious disease.

Acknowledgement:-

The authors wish to place their record of thanks to the authorities of Jamal Mohamed College (Autonomous), Trichy, for providing a good infrastructure facility.

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