African Journal of Fisheries science ISSN 2375-0715 Vol. 9 (2), pp. 001-006, September, 2021. Available online at www.internationalscholarsjournals.com © International Scholars Journals

Author(s) retain the copyright of this article.

Review article

# Algae as antioxidants and effective fish feed

Priyanka Mukherjee\* and Ruma Pal

Department of Botany, University of Calcutta, 35, Ballygunge Circular Road, Kolkata- 700 019, India.

#### Accepted 15 September, 2021

The oxidative stress in the body of an organism whether plant or animal leads to the production of several reactive oxygen and reactive nitrogen species abbreviated as ROS and RNS respectively. Algae, because of the presence of various biochemical compounds can act as a potent antioxidative agent and can thrive against the stressful conditions produced due to the oxidative stress. These secondary metabolites are high value compounds which can be efficiently used in the field of aquaculture as feed for different fishes. Thus in this review we have discussed the various compounds isolated from algae which allows the algal life to thrive in high oxidative stress as well as we have discussed different potential algal genera being used as feed for different fishes.

Key words: Antioxidant nature of algae, different bioactive compounds, algae based aquaculture feed.

## INTRODUCTION

#### **Review of literature**

The imbalance between the pro-oxidants and the antioxidants leads to the production of several Reactive Oxygen Species commonly called as ROS. These reactive oxygen species are mainly hydroxyl group (OH), superoxide anion  $(O_2)$ , hydrogen peroxide  $(H_2O_2)$  and peroxyl radicals. These reactive oxygen species in low concentration generally bring about minor changes in the normal cellular redox status, immune function and intracellular functioning of a particular organism. But high concentration of reactive oxygen species leads to the peroxidation of lipids, damages protein and DNA and gets involved with the cellular function leading to cell death by necrosis and cellular apoptosis (Halliwell, et al. 1997; Temple, 2000). Along with damage of the cellular components, ROS also plays an active role in regulation of signal transduction system, gene expression, activation of receptors and nuclear transcription factors (Packer, 2003). Algae are exposed to a combination of diverse environmental conditions which often may act as stress and lead to the production of different free radicals (Dykens, et al. 1992). Thereby, their elements of the photosynthetic apparatus are also being exposed to photodynamic damage regularly, because the major

constituent of the thylakoid membrane is polyunsaturated fatty acids (Sukenik, et al. 1993). But the absence of damage to algae in spite of being in proximity to the ROS suggests that these cells have protective antioxidative mechanisms and compounds which provide them shelter from harmful activity of ROS. The protective compounds present in algae are majorly polysaccharides, fattyacids, tannins, pigments, proteins and vitamins. The phlorotannins (compounds with polymerized phloroglucinol units) are also noteworthy antioxidants present in algae especially in Phaeophyceae. They find profound usage in broad therapeutic perspectives, such as anti-diabetes, antioxidation, radiation protection, anti-cancer, anti-HIV as well as anti-allergic activities (Dellabella, 2005; Rosa, et al. 2007; Zang, et al. 2008; Halliwell, et al. 2008). Other than polyphenols algae are also rich in peptides which are gaining interest because of their potent antioxdative nature (Je, et al. 2005; Mendis, et al. 2005; Qian, et al. 2008a,b). Thus these algal peptides can be easily implemented as alternative antioxidative supplements. Algae are also rich in phytophenolic compounds and pigments. The Rhodophyta (red algae) are a distinct eukaryotic lineage, which are rich in accessory photosynthetic pigments such as phycoerythrin, phycocyanin and allophycocyanins arranged in phycobilisomes. They are also efficient antioxidants. Thus as algae, have been documented to be rich sources of various bioactive compounds they can easily and safely be incorporated in various biomedical fields and food industry (Mukherjee, et al. 2018).

<sup>\*</sup>Corresponding author. Priyanka Mukherjee, Email: priyankamukherjee76@gmail.com.

In animal system the ROS bring about oxidative modification of DNA and proteins and other cellular molecules leading to the development of various range of common diseases such as cardiovascular disease, atherosclerosis, diabetes mellitus inflammatory conditions, and neurodegenerative disease such as Alzheimer's disease, Parkinson's disease and immune dysfunction (Rechardson, 1993; Halliwell, 2000;Young, et al. 2001), mutations and cancer (Byres, et al. 1995) and age related degenerative disease (Borek, 1993). Thus to stop the damage causing activity of ROS an efficient antioxidant is required.

The use of synthetic antioxidants has decreased nowadays because studies have shown that synthetic antioxidants act as promoters of carcinogenesis furthermore people today reject to use synthetic food additives frequently (Pratt, 1992; Grillo, et al. 1995; Wang, et al.1995). Thus these findings along with the general interest have promoted the need for finding out new alternative antioxidants from natural sources (Huang, et al. 2004). Sea weeds have found their usage since many years in the folk medicine for variety of remedial purposes (Chapman, et al. 1980).

Microalgae also play an important role as feed ingredients for fishes, because of their nutritive properties, vibrant pigment profiles and a high content of bioactive compounds with cytostatic, antiviral, anthelmintic, antifungal and antibacterial activities as detected in green, brown and red algae (Newman, et al 2003). Aquaculture or rearing of aquatic organisms under controlled conditions for commercial purposes date back to 6000 BC. The concept was first developed by Gunditimara people of Victoria, Australia where they first converted volcanic floodplains in the vicinity of Lake Condah into complex of channels and dams for capturing eel fish for consumption. Today, almost 50% of the world's trade comes from the aquaculture based industry. Fish culture in India is very popular and extensive. Along with the countries consumption, these fishes also enter the world market as a form of export. Intensive aquaculture is highly dependent on fish oil and fish meal as feed ingredients. Fish meal stands to be essential dietary sources of protein, amino acids, fatty acids (Tacon, 1993). To avoid different constrains in this field the usage of algae as a potent feed has found different openings. Several studies have revealed that different algae are being commonly used in the field of aquaculture as different fish feed are Chlorella, Dunaliella, Haematococcus, Ulva, Enteromorpha, Laminaria, Porphyra, Sargassum, Gracillaria and many more (Tabarsa, 2012).

This review deals with the different antioxidant contents of different algae and the use of different algae with high antioxidant properties in the field of aquaculture. **DIFFERENT ALGAE AND THEIR COMPOUNDS RE-SPONSIBLE FOR THEIR ANTIOXIDATIVE PROPER-**

#### TΥ

In recent years, functional foods have been noticed to gain more interest because of their ability to provide physiological benefits in addition to nutritional and energetic, as, for instance, antihypertensive, antioxidant or anti-inflammatory actions (Goldberg, 1996). A functional food can be defined as a food that benefits in one or more physiological functions, increases the welfare and/or decreases the risk of suffering from a particular disease. Along with the functional food the most upcoming field in the recent years are the nutraceutical aspects of natural products. People today are more interested or rather prefer natural products both as functional food as well as nutraceuticals. Today the natural sources which are being widely exploited for these purposes are plants, food by-products, fungi and algae. Algae nowadays have become a hot topic of discussion because of their high content of biochemical parameters such as polyunsaturated fatty acids (Cohen, et al. 199; Mahajan, et al. 1995),  $\beta$ -carotene and other pigments (antioxidants) (Bhat, et al. 2000), sulphated polysaccharides (antiviral), and sterols (antimicrobials).(Borowitzka, et al. 1988; Otles, et al. 2001; Xue, et al. 2002).

Several studies have shown that algae have developed an efficient defense system that helps them to survive under environmentally adverse conditions during evolution. This property has developed in algae due to the compounds present such as tannins, peptides, pheophytins polysaccharides and other pigments. Tannins are phenolic compounds that show antioxidant activity due to their high redox properties, which play an important role in absorbing and neutralizing free radicals thereby quenching singlet and triplet oxygen or decomposing peroxides. Studies of (Yasantha, et.al.2006) have shown that enzymatic hydrolysate from brown alga, Ecklonia cava collected from Jeju Island coast of Korea to have a high antiproliferative and antioxidative action. This algal enzymatic hydrolysates had efficient antioxidative action against the DPPH reagent as well as superoxide anions, hydrogen peroxides, hydroxyl radicals which act as the precursors of the singlet oxygen species, thereby indirectly inhibiting lipid peroxidation. This property was attributed to the algae by the various polyphenolic as well as polysachharide compounds present in it. The crude polysaccharide and crude polyphenolic fractions of the alga showed antioxidative action. Different studies have shown that in E. cava. it is the polyphenolic compounds including phlorotannins which remains to be majorly responsible for thriving against various oxidative stress and damages in algae (Reddy, et al. 2003).

Polyphenols have also been found to be widely present in different algae such as *Sargassum swartzii*, *Cystoseira myrica*, *Colpomenia sinuosa* (Sadati, et al. 2011).

Algal polyphenolic compounds apart from acting as a good antioxidant in various cell lines also show effective activity in delaying fish oil rancidity. This was first shown by Yan in 1996, where Phlorotannin isolated from *Sargassum kjellmanianum* could also protect fish oil from rancidity. This is due to the fact that polyphenols easily transfer a hydrogen atom to lipid peroxyl cycle and form the aryloxyl, which being incapable of acting as a chain carrier, couples with another radical thus quenching the radical process (Ruberto, et al. 2001).

Apart from the polyphenols the pigments also play a major role in antioxidative property of algae. Microalgae are rich sources of pigments comprising of mainly two main types of chlorophyll, chlorophyll a and chlorophyll,  $\beta$ -carotene, astaxanthin and lutein which are considered to be most important forms of 600 naturally occurring carotenoid for their potential applications in food additives for human

nutrition, nutraceuticals, nutritional supplements (Zhang, et al. 2014). Carotenoids are the only group of biologically active compounds that possess antibacterial, antiviral, antifungal, antioxidative, anti-inflammatory, and antitumor properties. Several algal species such as *Haematococcus pluvialis*, *Dunaliella salina*, *Chlorella sps*, *Scenedesmus sps*, *Spirulina platensis*, *Botryococcus braunii*, and *Diatoms* have gained worldwide fame in the commercial production of  $\beta$ -carotene, lutein, canthaxanthin, astaxanthin, and fucoxanthin (Zhang, et al. 2014).

The algal pigments such as taxanthin also play a very important role as free radical scavenger in algae such as *Haematococcus pluvialis* (Kobayashi, et al. 1997). Research work (Shaish, et al. 1993) has shown that the same mechanism also occurs in *Dunaliella bardawil*. Carotenoids such as astaxanthin act as scavenger of several active oxygen species such as free oxygen radical, hydrogen peroxide, peroxy radicals and hydroxyl radicals (HO) both *in vitro* and *in vivo* (Burton, 1989; Krinsky, 1989; Miki, 1991; Lim, et al. 1992; Lawlor, et al. 1995). Thus, astaxanthin, provides protection to the cytoplasm and lipid membranes (Krinsky, 1989). Therefore, this particular carotenoid is now being utilized as a pigment source in pisciculture (Benemann, 1992) and is also being investigated for medical applications because it is more antioxidative than b-carotene and vitamin E (Miki, 1991; Palozza, et al. 1992).

Other than the pigments algae are also rich in polysaccharide also show antioxidative activity. Different studies of maximum works with brown algae have been reported till date but only little information is available with Chlorophycean members.

## ALGAE AS FISH FEED ADDITIVES

Aquafarming today is also one of the most important businesses all over the world. Use of algal feed in fish aquaculture is well in practice for both edible and colored fishes for their nutritional constituents as well as pigment composition. Works of several authors have shown the usage of different algae such as *Porphyridium, Pavlova, Chaetoceros, Gracillaria, Palmaria* as efficient colour elicitors and growth enhancers in cichlid fish, rainbow trout, fish larvae, bivalve mollusks, black tiger prawns and several gastropods (Kop, et al. 2008 ;Sudaporn, et al. 2010). Several genera like *Skeletonema, Chaetoceros, Thalassiosira, Tetraselmis* and *Isochrysis* with long-chain polyunsaturated fatty acids are also well documented as fish feed ingredients or live feed (Borowitzka, 2005; Borowitzka, 2006; Chisti, 2006; Huntley, et al. 2007).

Studies have shown use of different algal genera, such as *Isochrysis, Pavlova, Chaetoceros, Gracillaria, Palmaria, Haematococcus, Arthrospira, Dunaliella, Chlorella, Chlorococcum* etc. to play important role as food ingredients for fishes as they are rich sources of several bioactive compounds which help them (Guerin, et al. 2003). The rich contents of several pigments especially carotenoids have made algae popular amongst fish breeders as feed ingredients. Studies of (Chatzifotis, et al. 2005) revealed that from the green algae *Haematococcus pluvialis*,  $\beta$ ,  $\beta$ -carotene and lycopene can be extracted and used as an efficient alternative to

synthetic pigments in red porgy. A green algae Ulva containing good vitamin, mineral and pigment profiles have found its usage as a potent feed ingredient for a wide range of fish species (Mustafa, et al. 1995; Wassef, et al. 2005; Valente, et al. 2006). Incorporation of Cladophora, Chaetomorpha, Pithophora, Rhizoclonium, Spirulina, Leptolyngbya etc. in fish feed enhanced the carotenoid content as well as immunity in goldfish as well as catfish respectively (Mukherjee, et al. 2019, Promya, et al. 2011). Several studies have shown the usage of different algae such as Leptolyngbya valderiana, L. tenuis, Arthrospira maxima, Navicula minima, Nostoc ellipsosporum, Ulva, Cytoseira, Chlorella, Porphyridium, Isochrysis, Pavlova, Chaetoceros, Gracillaria, Palmaria, Isochrysis etc. as efficient color elicitors in cichlid fish, golden gourami, wag swordtail, orange molly pink zebra, rainbow trout, tetras, prawns, Nile tilapia etc. Therefore incorporation of varied algae in the fish meal diet is cost effective way is a major challenge for improvement of both the production rate as well as their immunity (Mukherjee, et al. 2015; Deventor, et al. 1996; Gupta, et al. 2007; Sudaporn, et al. 2010).

### CONCLUSION

Researches through years have thus shown algae to be efficient multitasking organisms. They not only play major role in pollution controlling system but also act as efficient antioxidant against oxidative stress management. The high biochemical contents have always proved algae to be the best natural resource ever to be found. Thus because of these properties algae would find immense usage in medicinal field as well as in the field of aquaculture.

#### REFERENCES

- Bhat VB, Madyastha KM (2000). C-Phycocyanin: A potent peroxyl radical scavenger in vivo and in vitro. Biochem Biophy Resrch Comm. 275: 20-25.
- Borek C (1993). Molecular mechanisms in cancer induction and prevention. Env Health Pers. 101: 151-160.
- Carolina T. Miranda, Roberta F. Pinto, Daniel V. N. de Lima, Carolina V. Viegas, Simone M. da Costa, Sandra M. F. O. Azevedo (1988). Microalgae Lipid and Biodiesel Production: A Brazilian Challenge. Am J Plant Sci. 6 (15): 1.
- Borowitzka MA (2005). Culturing of microalgae in outdoor ponds. [Book auth.] RA Andersen. Algal Cult Tech, London: Elsevier, 14.
- 5. Borowitzka MA (2006). Biotechnological and environmental applications of microalgae.
- Burton GW (1989). Antioxidant action of carotenoids. J Nutr. 119:109-111.
- Byres T, Guerrero N (1995). Epidemiologic evidence for vitamin C and vitamin E in cancer prevention. Amer Journ Clin Nut. 62: 1385-1392.

- 8. Chapman VJ, Chapman DJ (1980). Seaweeds and their uses. Chapman and Hall: London.
- Chatzifotis S, Pavlidis M, Jimeno CD, Vardanis G, Sterioti A, Divanach P (2005). The effect of different carotenoid sources on skin coloration of cultured red porgy (*Pagrus pagrus*). Aquacult Resrch. 36(15):1517-1525.
- 10. Chisti Y (2006). Microalgae as sustainable cell factories. Environ Eng Manag J. 5(3): 261-274.
- Cohen Z, Vonshak A (1991). Fatty acid composition of Spirulina and Spirulina-like cyanobacteria in relation to their chemotaxonomy. Phytochem. 30: 205-206.
- 12. Dellabella M, Milanese G, Muzzonigro GJ (2005). Urol. 174: 167.
- 13. Deventer B, Heckman CW (1996). Effects of prolonged darkness on the relative pigment content of cultured diatoms and green algae. Aqu Sci. 58: 241-252.
- Dykens JA, Shick JM, Benoit, Buettner GR, Winston GW (1992). Oxygen radical production in the sea anemone *Anthopleura elegantissima* and its endosymbiotic algae. J exp Biol. 168: 219-241.
- Goldberg I (1996). Functional foods. Designer foods, pharmafood, nutraceuticals Londres, Gran Bretan: A Chapman and Hall. 3.
- 16. Grillo CA, Dulout FN (1995). Cytogenetic evaluation of butylated hydroxytoluene. Mutat Res. 345: 73-78.
- Gupta SK, Jha AK, Pal AK, Venkateshwarlu G (2007). Use of natural carotrenoids for pigmentation in fish. Nat Prod Rad. 6 (1): 46-49.
- Guerin M, Huntley ME, Olaizola M.(2003). Haematococcus astaxanthin: Applications for human health and nutrition. Trends Biotechnol.2: 210-216.
- 19. Halliwell B (2000). The antioxidant paradox. The Lancet. 355: 1179-1180.
- 20. Halliwell B (2008). Free Radical Biol. Med. 11.
- 21. Halliwell B (1997) Antioxidants and human disease. A general introduction. Nutr rev. 55: 44-52.
- Huang HL, Wang BG (2004). Antioxidant capacity and lipophilic content of seaweeds collected from the Qingdao coastline. J Agric Food Chem. 58: 4993-4997.
- Huntley ME, Redalje, DJ (2007). CO<sub>2</sub> Mitigation and renewable oil from photosynthetic microbes: A new appraisal. Mitig Adapt Strateg Glob Chang. 12(4): 573-608.
- Je JY, Park PJ, Kim SK. (2005). Antioxidant activity of a peptide isolated from Alaska Pollack (*Theragra chalcogramma*) frame protein hydrolysate. Food Res Int. 38: 45-50.

- Kobayashi M, Kakizono T, Nagai S. (1993) Enhanced carotenoid biosynthesis by oxidative stress in acetate-induced cyst cells of a green unicellular alga, Haematococcus pluvialis. Appl Environ Microbiol. 59: 867-873.
- 26. Kop A, Durmaz Y (2008). The effect of synthetic and natural pigments on the colour of the cichlids (*Cichlasoma severum* sp.,Heckel 1840). Aquacult Int. 16: 117-122.
- 27. Krinsky NI (1989). Antioxidant functions of carotenoids. Free Radic Biol Med. 7: 617-635.
- Lawlor SM, O'Brien NM (1995). Astaxanthin: Antioxidant effects in chicken embryo fibroblasts. Nutr Res. 15: 1695-1704.
- Mahajan G, Kamat M (1995). C-Linoleic acid production from *Spirulina platensis*. Appl Micro Biotech. 43: 466-469.
- Mendis E, Rajapakse N, Byun HG, Kim SK (2005). Investigation of jumbo squid (Dosidicus gigas) skin gelatin peptides for their in vitro antioxidant effects. Life Sci. 77: 2166-2178.
- 31. Miki W (1991). Biological functions and activities of animal carotenoids. Pure Appl Chem. 63: 141-146.
- 32. Mustafa GM, Nakagawa (1995). A review: Dietary benefits of algae as an additive in fish feed. Isr Journ Aquacult Barni. 47:155-162.
- Mukherjee P, Nandi C, Khatoon N, Pal R (2015). Mixed algal diet for skin colour enhancement of ornamental fishes. Journ Alg Bio Utili. 6 (4): 35-46.
- Mukherjee P, Pal R (2018). Antioxidative potentials of four green filamentous algae of Indian Sunderbans. Journ Alg Bio Utili. 9(4): 1-10.
- 35. Mukherjee P, Gorain PC, Paul I, Bose R, Bhadoria PBS, Pal R (2019). Investigation on the effects of nitrate and salinity stress on the antioxidant properties of green algae with special reference to the use of processed biomass as potent fish feed ingredient. Aqua Int. 5 (27) : 1-24.
- Newman DJ, Cragg GM, Snader KM (2003). Natural products as source of new drugs over the period 1981-2002. Journ Nat Prod. 66: 1022-1037.
- O"tles S, Pire R (2001). Fatty acid composition of *Chlorella and Spirulina* microalgae species. Journ AOAC Int. 84: 1708–1714.
- Packer L, Cadenas E, Davies KJA (2008). Free Radical Biol. Med. 44: 123.
- Palozza P, Krinsky NI (1992). Astaxanthin and canthaxanthin are potent antioxidants in a membrane model. Arch Biochem Biophys. 297: 291-295.

- 40. Pratt DE (1992). Natural antioxidants from plant material. In: Huang MT, Ho CT &Lee CY (Editors), Phenolic compounds in food and their effects on health. II.American Chemical Society, Washington. ACS Symposium Series. 507: 54-71.
- 41. Promya J, Chitmanat C (2011). The effects of *Spirulina platensis* and *Cladophora* algae on the growth performance, meat quality and immunity stimulating capacity of the African sharptooth catfish (*Clarias gariepinus*). Int J Agric Biol. 13:77-82.
- 42. Qian ZJ, Jung W K, Kim SK. (2008b). Free radical scavenging activity of a novel antioxidative peptide purified from hydrolysate of bullfrog skin, Rana catesbeiana Shaw. Bioresour Technol. 99: 1690-1698.
- 43. Qian ZJ, Jung WK, Byun HG, Kim SK (2008a). Protective effect of an antioxidative peptide purified from gastrointestinal digests of oyster, *Crassostrea* gigas against free radical induced DNA damage. Bioresour Technol. 99: 3365-3371.
- Rechardson SJ (1993). Free radicals in the genesis of Alzheimer's disease. Annal New York Acad Sci. 695:73-76.
- 45. Rosa A, Deiana M, Atzeri A, Corona G, Incani A, Melis MP, Appendino GM, et al. (2007). Chem.-Biol. Interact.165: 117.
- Ruberto G, Baratta MT, Biondi DM, Amico V (2001). Antioxidant activity of extracts of the marine algal genus *Cystoseira* in a micellar model system. Journ Appl Physio. 13: 403-407.
- 47. Sadati N, Khanavi M, Mahrokh A, Nabavi SMB, Sohrabipour J, Hadjiakhoondi A (2011).Comparison of antioxidant activity and total phenolic antioxidant activities of marine algae 203 Contents of some persian gulf marine algae. Journ Med.Plants. 10 (37): 73-79.
- Sukenik A, Zmora O, Carmeli Y. (1993). Biochemical quality of marine unicellular algae with special emphasis lipid composition: II. *Nannochloropsis* sp Aquacult. 117: 313–326.

- 49. Sudaporn TK, Yuwadee P (2010). Effect of replacing fishmeal with *Spirulina* on growth carcass composition and pigment of Mekong giant catfish. Asian Journ Agricult Sc. 2(3): 106- 110.
- 50. Temple N J (2000).Antioxidants and disease. More question than answers. Nutr Res. 2: 449 459.
- 51. Tabarsa M, Lee SJ, You SG (2012). Structural analysis of immunostimulating d polysaccharides from Ulva pertusa. Carbohydr. Res. 361:141-147.
- 52. Tacon AGJ (1993). Feed ingredients for warm water fish. Fish meal and other processed feedstuffs, FAO Fisheries Circular. 856:64.
- 53. Valente LMP, Gouveia A, Rema P, Matos J, Gomes EF, Pinto IS (2006). Evaluation of three seaweeds Gracilaria bursa-pastoris, Ulva rigida and Gracilaria cornea as dietary ingredients in European sea bass Dicentrarchus labrax juveniles. Aquacult. 252: 85-91.
- Wang X, Witschi H (1995). Mutations of the Ki-ras protooncogene in 3-methylcholanthrene and urethaneinduced and butylated hydroxytoluene-promoted lung tumors of strain A/J and SWR mice. Can Res. 91: 33-39.
- 55. Wassef EA, El-Sayed AFM, Kandeel KM, Sakr EM (2005). Evaluation of *Pterocladia* and *Ulva* meals as additives to gilthead seabream *Sparus aurata* diets. Egypt Journ Aquat Res. 31:321-332.
- Xue C, Hu Y, Saito H, Zhang Z, Li Z, Cai Y (2002). Molecular species composition of glycolipids from *Spirulina platensis*. F Chem. 77: 9-13.
- 57. Young IS, Woodside JV (2001). Antioxidants in health and disease. J Clin Pathol. 54(3): 176-186.
- Zhang R, Kang KA, Piao M J, Ko DO, Wang ZH, Lee IK, Kim BJ, et al. (2008). Eur. J. Pharmacol.591: 114.
- 59. Zhang X, Rong J, Chen H, He C, Wang Q (2014). Current Status and outlook in the application of microalgae in biodiesel production and environmental protection. Front Ener Resrch 2.